Do not follow where the path may lead.

Go instead where there is no path

And leave a trail.

R. W. Emerson

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Development of tools for the *in vitro* conservation and modulation of saponin production of four medicinal *Maesa* species

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List of abbreviations and acronyms

2,4-D	2,4-dichlorophenoxyacetic acid
αAS	α-amyrin synthase
ABA	Abscisic acid
AFLP	Amplified length fragment
AR	Adventitious root
AS	Acetosyringone
βAS	β-amyrin synthase
B5	Gamborg B5 (macro and micro elements)
BA	Benzyladenine
BSA	Bovine serum albumin
BTH	Benzothiadiazole
CAM	Chick chorioallantoic membrane
cDNA	Complementary DNA
CDPK	Calcium-dependent protein kinase
CFW	Calcofluor white
CS	Cell suspension
DDS	Dammarenediol synthase
DIECA	Diethyldithiocarbamate
DMAPP	Dimethylallyl diphosphate
DMSO	Dimethyl sulfoxide
d.w.	Dry weight
EST	Expressed sequence tag
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FDA	Fluorescein diacetate
FPP	Farnesyl diphosphate
FPS	Farnesyl diphosphate synthase

GA3	Gibberellic acid
GFP	Green fluorescent protein
GPP	Geranyl diphosphate
GPS	Geranyl diphosphate synthase
GT	Glycosyl transferase
HEJ	2-hydroxyethyl jasmonate
HPLC	High performance liquid chromatography
HR	Hairy root
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IC_{50}	50% inhibitory concentration
IPP	Isopentenyl diphosphate
Kin	Kinetin
KM	Kao and Michayluck (macro and micro elements)
LC	Liquid chromatography
LS	Loading solution
MA	*Maesa argentea*
MB	*Maesa balansae*
MeJA	Methyl jasmonate
MEP	Methyl-erythritol
MES	2-(N-morpholino)ethanesulfonic acid
ML	*Maesa lanceolata*
MP	*Maesa perlarius*
MS	Mass spectroscopy
	Murashige and Skoog (macro and micro elements)
	Multisizer
MVA	Mevalonate
NAA	α-naphthalene acetic acid
NMR	Nuclear magnetic resonance
NPA	Naphthylphtalamic acid
OE	Over expression
ORF	Open reading frame
OD	Optical density

PAI	Primula acid I
PCR	Polymerase chain reaction
PCV	Packed cell volume
PEG	Polyethylene glycol
PVS2	Plant vitrification solution
qPCR	Quantitative PCR
R_f	Retention factor
Ri	Root inducing
RNAi	RNA interference
RT	Retention time
RT-PCR	Reversed transcriptase PCR
SA	Salicylic acid
Sad	Saponin-deficient
SE	Squalene epoxidase
SEC	Squalene-2,3-epoxide cyclase
SH	Schenk and Hildebrandt (macro and micro elements)
SS	Squalene synthase
TIB	Temporary immersion bioreactor
T-DNA	Transfer DNA
TDZ	Thidiazuron
Ti	Tumor inducing
TLC	Thin layer chromatography
WPM	Woody plant medium (macro and micro elements)
YE	Yeast extract
Zea	Zeatin

Scope

A continuous ingression of new diseases is threatening humans, animals and plants. In addition, there is an inadequacy to cope with natural drug resistance in cancer cells and infectious micro-organisms, toxicity and undesirable side effects associated with drugs currently used. Consequently, only one third of all human diseases can be treated properly and the pharmaceutical industry is permanently searching for novel molecules with new or superior activities. In recent years, there was a renewed interest in the use of plants as a source of drug discovery because the chemical diversity in plants is much larger than any chemical library made by humans. This thesis focuses on four *Maesa* species that are used in Asian and African folk medicine and have been reported to produce triterpene saponins as the major active substances. *Maesa* saponins may have an important role in the future treatment of leishmania and cancer. However, the saponins have side-effects, such as haemolytic activity, and are therefore not readily applicable. In addition, *Maesa* species are tropical plant species that are difficult to cultivate in a moderate climate like in Belgium. This could be a serious disadvantage for commercial production, when a continuous supply of material needs to be ensured. Therefore, the first goal of this thesis was to establish protocols for efficient *in vitro* cultivation and propagation of four *Maesa* species. These cultures could also be used for further *in vitro* techniques, such as protoplast isolation or genetic transformation. To evaluate the saponin production in different *in vitro* cultures and regenerated plantlets, we had to optimize the technique for saponin extraction and thin layer chromatography (TLC). The second goal of this PhD was to modulate the saponin production in *Maesa* species. Previous studies showed that small structural changes of the saponins could have a drastic effect on their activity and toxicity. Therefore we intended to produce novel saponins with different or new biological activities. Since chemical synthesis of saponins is not feasible and no genomic information is available for *Maesa* species, we attempted two semi-rational combinatorial biosynthesis approaches. For the first method, putative *Maesa* saponin biosynthesis genes were silenced. No transformation techniques were at our disposal for *Maesa*, therefore we first had to work out a transformation protocol. These studies were performed within the framework of a larger project, COMBIPLAN or COMbinatorial BIosynthesis in PLANts. A second method was based somatic hybridization of different *Maesa* species. For this purpose, techniques for protoplast isolation, fusion and regeneration needed to be developed.

In summary, the overall scope of this thesis was to establish a platform with different tools for the study and modulation of saponin biosynthesis in four medicinal *Maesa* species.

CHAPTER 1 INTRODUCTION ON SAPONINS AND MAESA SPECIES

Parts of this chapter have been published as:

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1.1 Triterpene saponins

Saponins are naturally occurring surface-active glycosides. The name 'saponin' is derived from the Latin word *sapo*, which means 'soap' and reflects their property to form stable, soap-like foams in aqueous solutions (Hostettmann and Marston 1995). It is a diverse group of terpenoids characterized by their structure. Saponins are composed of a steroidal or triterpenoid aglycon and one or more sugar chains. A plant might contain several dozen saponins with closely related structures (Vincken et al. 2007). This structural diversity is the basis of their numerous physicochemical and biological properties which are exploited in many traditional and industrial applications.

Recent research has established saponins as the active components in many herbs and plants used in traditional medicine (Liu and Henkel 2002). A well known example is *Panax ginseng*, one of the most valuable oriental herbs (Liang and Zhao 2008; Wu and Zhong 1999). Dried ginseng roots are since ancient times used as a healing drug and health tonic in many Asian countries. Recently, ginseng has been increasingly used for different purposes and the medicinal value of ginseng is of vast interest worldwide. Extensive effort is still being put into investigating its pharmacological effects (Dang et al. 2009; Jia and Zhao 2009; Jia et al. 2009; Lu et al. 2009; Luo and Luo 2009).

Despite all the opportunities, the commercial production of saponins in plants is still facing many problems, such as low yields, limited availability of natural resources and the lack of successful classical breeding techniques. Because of that, much research effort is being put in finding a way to enhance the production of the saponins or driving the expression toward one interesting saponin.

1.1.1 Biosynthesis of triterpene saponins

Terpenes represent the largest family of natural compounds, with more than 30,000 entities being structurally identified (Sacchettini and Poulter 1997). They are derived from units of isoprene, which has the molecular formula $(C_5H_8)_n$. Most terpenes are classified by the number of C_5 isoprene units that they contain. The classes are: hemiterpenes consisting of a single C_5 unit, monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}), carotenoids (C_{40}) and polyterpenes consisting of long chains of many isoprene units.

Terpene biosynthesis is a complicated process mediated by two biosynthetic pathways; the mevalonate (MVA) and methyl-erythritol (MEP) pathway (Eisenreich et al. 1998). Both pathways lead to the formation of isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), which are the precursors from which all terpenes are formed (Fig 1-1). Head-to-tail addition of IPP and DMAPP leads to formation of geranyl diphosphate (GPP) and this step is mediated by GPP synthase (GPS). FPP synthase (FPS) is responsible for the addition of one IPP unit to GPP. Further on, squalene synthase (SS) will mediate the condensation of 2 FPP units for the synthesis of squalene, which is the precursor for both steroidal and triterpene saponins (Dubey et al. 2003) (Fig 1-1). Subsequently, squalene is oxidized to squalene-2,3-epoxide (also referred to as oxidosqualene). Squalene-2,3-epoxide is the common starting point for cyclization reactions in triterpene saponin biosynthesis (Fig 1-2) and is converted to cyclic derivatives which create a carbenium ion that can undergo several types of cyclization reactions. The type of cyclase that is involved in the cyclization reaction determines the skeleton that is formed. Many different kinds of cyclases have been described and their mechanisms of action are well documented; the most important ones are dammarenediol synthase (DDS), β-amyrin synthase (βAS) and α-amyrin synthase (αAS), which will give rise to dammarane, oleanane and ursane type of saponins, respectively (Shibuya et al. 2009; Vincken et al. 2007).

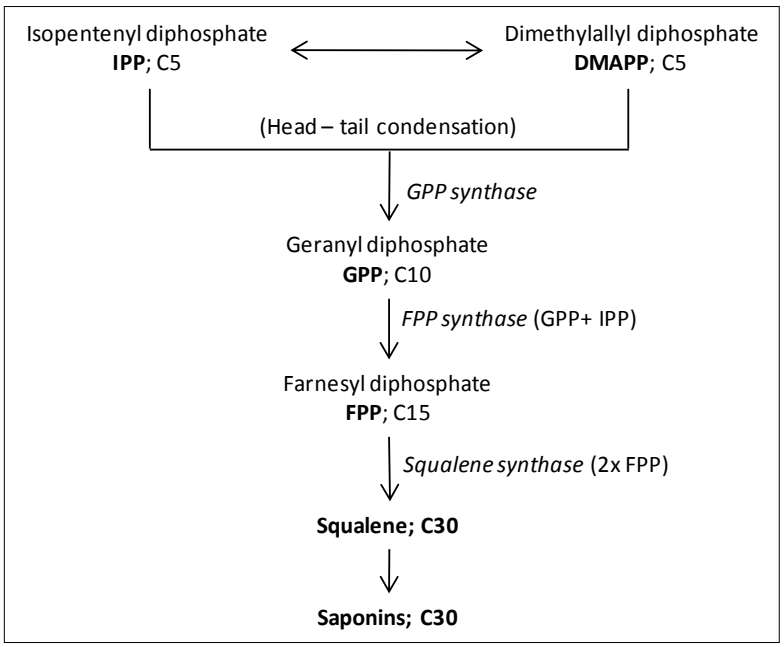


Fig 1-1 Synthesis of the saponin backbone from isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (adapted from Dubey et al. (2003))

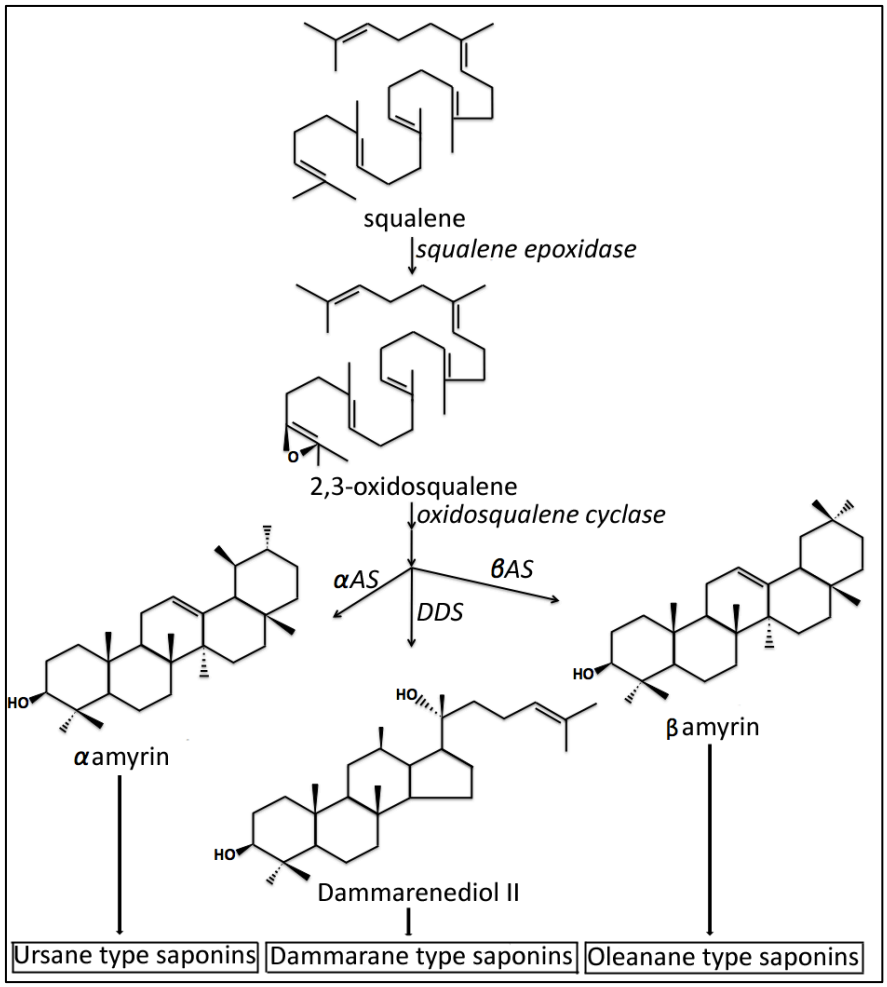


Fig 1-2 Synthesis of different classes of triterpene saponins from the common precursor squalene. Squalene is oxidized to squalene-2,3-epoxide, which is the common starting point for both triterpene and steroidal saponin synthesis. The type of cyclase that is involved in the cyclization of squalene-2,3-epoxide will determine the skeleton that is formed. Cyclization via a *chair-chair-chair* conformation will lead to triterpene saponins; cyclization via a *chair-boat-chair* conformation will result in steroidal saponins.

Following cyclization, further diversity is obtained by modification of the products through oxidation, hydroxylation, glycosylation and other substitutions mediated by cytochrome P450-dependent monooxygenases, glycosyltransferases and other enzymes. However, not much is known about the identity of the enzymes required for these reactions.

One common feature shared by all saponins is the presence of a sugar chain attached to the aglycon. Glycosylation is particularly important as the sugar chain is critical for the biological activity in several saponins. The oligosaccharide chains are likely to be synthesized by sequential addition of single sugar residues to the aglycon but there is little experimental data about the mechanism of triterpenoid glycosylation.

1.1.2 Medicinal properties of saponins

Saponins are considered to be the key ingredients in traditional medicines and are responsible for most of the observed biological effects (Francis et al. 2002; Liu and Henkel 2002; Sparg et al. 2004). For example, ginseng root is one of the most important traditional oriental medicines and triterpene saponins, ginsenosides, were proven to be the major constituents (Jia and Zhao 2009; Lu et al. 2009). Also the genus *Bupleurum* is often used in Asian traditional medicines to treat different diseases. Dried roots of *Bupleurum fruticescens* are used to treat disorders associated with inflammation. The main anti-inflammatory compounds were found to be saponins (Just et al. 1998). The saponin producing plants *Allium chinense* and *A. macrostemon* are used in traditional practices for treatment of chest pain, stenocardia (angina pectoris) and cardiac asthma (Baba et al. 2000).

Table 1-1 gives an overview of the most important medicinal properties that are assigned to triterpene saponins. In addition, examples of plants producing these saponins are given.

Table 1-1 Overview of most important medicinal activities of triterpene saponins and examples of plants producing saponins with the given activity

Medicinal activity	Plant species	References
Anti-inflammatory	*Bupleurum fruticescens, Aesculus hippocastanum, Kalopanax pictus, Lonicera japonica, Panax ginseng*	(Just et al. 1998), (Sirtori 2001), (Li et al. 2002), (Kwak et al. 2003), (Kim et al. 1998)
Antifungal	*Maesa lanceolata, Panax notoginseng, Colubrina retusa, Hedera colchica, Chenopodium quinoa, Phytolacca tetramera*	(Sindambiwe et al. 1998), (Ma et al. 1999), (Li et al. 1999), (Mshvildadze et al. 2000), (Dini et al. 2002), (Escalante et al. 2002)
Antibacterial	*Cussonia spp., Clematis ganpiniana, Acacia cochliancantha, Euclea crispa, Astragalus melanophrurius, Hedyotis nudicaulis, Colubrina retusa*	(De Villiers et al. 2010), (Ding et al. 2009), (Manriquez-Torres et al. 2007), (Magama et al. 2003), (Calis et al. 1997), (Konishi et al. 1998), (ElSohly et al. 1999)
Antiparasitic/ antileishmanial	*Maesa balansae, Hedera helix, Glinus oppositifolius*	(Germonprez et al. 2004), (Delmas et al. 2000), (Traore et al. 2000)
Cytotoxic/ antitumor	*Aralia dasyphylla, Panax ginseng, Hedera colchica, Silene fortunei, Acacia tenuifolia, Pittosporum viridiflorum*	(Xiao et al. 1999), (Shibata 2001), (Barthomeuf et al. 2002), (Gaidi et al. 2002), (Seo et al. 2002)
Antiviral	*Maesa lanceolata, Tieghemella heckelii, Camellia sinensis, Aesculus chinensis*	(Apers et al. 2001), (Gosse et al. 2002), (Hayashi et al. 2000), (Yang et al. 1999)
Antioxidant	*Panax vietnamensis, Panax ginseng, Entada rheedii*	(Huong et al. 1998), (Tung et al. 2010), (Nzowa et al. 2010)
Vaccine adjuvant	*Polygala senega, Quillaja saponaria, Pulsatilla chinensis*	(Estrada et al. 2000), (Barr et al. 1998), (Sun et al. 2010)
Hepatoprotective	*Panax notoginseng, Panax vietnamensis, Hedychium coronarium*	(Yoshikawa et al. 2003), (Le Tran et al. 2001), (Nakamura et al. 2008)

1.1.3 Role of saponins in plants

As mentioned in the previous paragraph, many research articles describe the identification of saponins in plants with a focus on their biological activities. Amongst others, saponins have been reported to have antimicrobial, virucidal and insecticidal action (Francis et al. 2002; Sparg et al. 2004). In this view, saponins could be considered as a part of the plant defense mechanism and can be classified in a large group of protective molecules, namely **phytoprotectants** (Gonzalez-Lamothe et al. 2009; Morrissey and Osbourn 1999).

Several studies indicate that antimicrobial saponins confer protection against disease and this is studied in detail in oat. Avenacins are oat root saponins that are important for broad-spectrum resistance to soil-borne pathogens, including *Gaeumannomyces graminis* which causes 'take-all' disease in cereals (Papadopoulou et al. 1999). Saponin-deficient mutants (*sad* mutants) showed compromised resistance to several pathogens, indicating that avenacins provide a chemical defense against pathogen attack (Mylona et al. 2008; Trojanowska et al. 2001). In addition, *G. graminis* has the capacity to detoxify the major avenacin, avenacin A-1. Fungal mutants lacking the saponin-detoxifying enzyme, avenacinase, showed increased sensitivity to avenacin A-1 and were no longer able to infect oat (Bowyer et al. 1995). These studies imply that the antimicrobial saponins in oat act in defense-related processes and strengthen the relevance of these compounds as biotechnological weapons against pathogen infection.

1.1.4 Biological action of saponins

The exact role and mode of action of saponins in plants is not yet elucidated, though; the effect of purified saponins on humans, animals, bacteria and viruses has been studied in more detail. A large number of the biological effects of saponins, such as bactericidal, virucidal and fungicidal activity, have been ascribed to their action on membranes, specifically their ability to make pores in the membranes (Francis et al. 2002). Most likely this results from an affinity of the saponins for membrane sterols, mainly cholesterol (Bangham et al. 1962). In humans and animals, the affinity of saponins for sterols leads to a strong haemolytic action but has also implications for nutrient uptake through the intestinal membrane and the cholesterol metabolism. In addition, saponins can form sparingly digestible complexes with proteins and therefore influence the nutritive value of diets. Many more activities are assigned to saponins, some of them contradictory though, for example, saponins are found to have an effect on

endogenous insulin levels, on animal reproduction, on the human immune system, on malignant cells and on glucocorticoid levels.

1.1.5 Saponin production in *in vitro* cultures

Triterpenoid saponins have complex structures, making chemical synthesis an economically uncompetitive option for large-scale production. Therefore, the current supply of saponins is mostly extracted from plants grown in the field, which is also accompanied with difficulties. The production and harvesting process is time consuming and low yielding, in addition, the yield of saponins is dependent on geographical and seasonal variation. For example, *Panax* seeds must undergo a stratification process with exposure to cool-warm cycles in the preceding winter-summer seasons to promote zygotic embryo maturation and overcome dormancy. During the 18 month period of stratification and germination, there is a risk of infection by pathogens and abiotic stress which can reduce the viability of the seeds (Punja et al. 2004). After germination, it takes a minimum of 4 years before roots can be harvested and a 6-year-old root yields only about 90g fresh weight (Choi et al. 2003). In addition, there are no horticulturally improved varieties, so the cultivated ginseng plants are morphologically and genetically heterogeneous, which is reflected in the variable sizes, shapes and overall appearance of the marketed roots (Punja et al. 2004). An additional problem for many saponin-producing plants is that they are often endangered and protected in some areas (Okrslar et al. 2007).

Plant cell and tissue cultures have been proposed as alternatives for the production of pharmaceutically interesting saponins. These *in vitro* cultures could overcome many problems associated with saponin extraction from field-grown plants; production is not dependent on geography and season, product quality and yield are generally established and clonal propagation methods can be used to overcome germination and plant heterogeneity issues (Wu and Zhong 1999). In addition, *in vitro* cultures are highly suitable to manipulate and improve the production of desired compounds through the use of biotechnological methods, which will be discussed below. However, there can be problems with the stability, growth rate and scaling up of *in vitro* cultures. So far, only ginsenosides from *Panax ginseng* are produced commercially from cell suspension cultures (Paek et al. 2009). Because of the high cost associated with tests on a larger scale, mainly private efforts are deployed to establish cell culture technology as a means to produce medicinal compounds.

Langhansová et al. (2005) established cell suspensions and adventitious root cultures of *Panax ginseng* and compared the growth and saponin production in Erlenmeyer flasks with large-scale bioreactors. As

well the total ginsenoside content as the production of particular ginsenosides differed in the different tissue cultures and cultivation systems tested. In adventitious root cultures, the particular ginsenoside profile was similar to that in native roots. However, the total content of saponins was only 1.8% of dry mass in Erlenmeyer flasks and 1.5% in a bioreactor, which was lower than the total content in native roots (3.3% of dry mass). *Panax ginseng* cells produced very high yields of total saponins, 1.3% and 4.3% for callus and cell suspension in bioreactor respectively. In the case of cell suspensions, a complete different profile of individual saponins was found, with very high production of the ginsenosides Rb1 and Rg1. Therefore, this system would be better for the production of a subselection of compounds. Also, due to the less defined differentiation status of the plant cells in the culture, there were less problems with the extraction of the desired compounds (Langhansova et al. 2005). Also for the medicinal plant *Primula veris* a comparison was made between different tissue and cell cultures. The main saponin in *P. veris*, primula acid I (PAI), is produced in the roots. The average content of PAI in *in vitro* seedlings, roots from micropropagated plants and adventitious root cultures was 1.5 to 2 times lower than in roots of plants grown in the soil. In callus and suspension cell cultures, the content was 8 times lower. Despite the lower saponin content, adventitious root cultures are a valuable alternative for the production of PAI, as *P. veris* in a highly endangered and protected plant species (Okrslar et al. 2007). *In vitro* cultures for the production of saponins were established for *Centella asiatica* (Mangas et al. 2008) and *Gypsophila paniculata* (Herold and Henry 2001). For both plant species, a correlation between saponin production and expression of key enzymes in the saponin biosynthesis was found.

For *Panax quinquefolius* (American ginseng) an *in vitro* propagation protocol through somatic embryogenesis was optimized. Embryogenic callus was induced on leaf and stem explants within two weeks. Suspension cultures were established by transferring embryogenic callus to liquid medium. Globular somatic embryos from these cultures were germinated with a success rate of 50-60%. Shoot and root development could be enhanced through the addition of gibberellic acid and 6-benzylaminopurine. Based on thin layer chromatography (TLC) analysis it could be concluded that ginsenoside profiles in seed-derived plants were identical to those in plantlets derived from tissue culture. This efficient method for multiplication of American ginseng could be used to avoid problems associated with seed stratification, germination and heterogeneity of *Panax* plants (Punja et al. 2004).

Most callus and cell suspension cultures described are grown in medium with exogenously applied auxin. However, auxin can have a negative impact on the biosynthesis of specific classes of secondary metabolites like anthraquinones (Arroo et al. 1995). A solution to this problem would be the production

of callus and cell cultures without exogenous application of synthetic auxins (Choi et al. 2003; Choi et al. 1998; Choi et al. 1999).

1.1.6 Elicitation of saponins in *in vitro* cultures

Plant cell culture technology is a promising alternative to field cultivation for the production of complex plant-specific metabolites, like saponins. However, low production titer could prevent commercial application.

Saponins are phytoprotectants which are either produced upon a stimulus conveyed by the pathogen or produced in a developmentally controlled fashion. Inducible phytoprotectants are known as **phytoalexins**, while constitutive phytoprotectants are called **phytoanticipins**. Phytoanticipins occur constitutively in healthy plants before challenge by microorganisms or other stresses. Some phytoanticipins are found on the plant surface; others are sequestered as preformed compounds in vacuoles or organelles and released through a hydrolyzing enzyme after pathogen challenge (Gonzalez-Lamothe et al. 2009). In contrast, phytoalexins are not present in healthy plants but are synthesized in response to pathogen attack or stress as part of the plant defense response and are restricted to the tissue colonized by the fungus and the cells surrounding the infection site (Morrissey and Osbourn 1999). These defense responses can be activated through a signal transduction pathway via recognition of an **elicitor** by receptors located in the plasma membrane and formation of secondary messengers, such as jasmonates, ethylene and salicylic acid, which in turn activate the expression of defense genes, including genes that encode for enzymes catalyzing the formation of secondary metabolites (Vasconsuelo and Boland 2007).

Elicitors

The term 'elicitor' is very general and refers to chemicals from various sources, biotic or abiotic, as well as physical factors, that can trigger a response in living organisms resulting in accumulation of secondary metabolites.

Methyl jasmonate (MeJA) is a widely used elicitor which modulates many physiological events in higher plants, such as defense responses, flowering and senescence and because of that is regarded as a new class of phytohormones. MeJA and its derivatives have been proposed to be key signaling compounds in the process of elicitation leading to the accumulation of secondary metabolites (Creelman and Mullet

1997). Recently, unconventional synthetic elicitors such as 2-hydroxyethyl jasmonate (HEJ) were also found to be very powerful in eliciting plant secondary metabolites in cell cultures (Hu and Zhong 2008). Salicylic acid (SA) is another elicitor that is widely studied as a stress signaling molecule involved in pathogen responses in plants. The importance of salicylic acid in the signal transduction pathway of disease resistance has been well documented (Raskin 1992). MeJA and SA are the most important elicitors used as inducers of triterpene saponin production. To a lesser extent also yeast extracts, phytohormones and heavy metals are used.

Changes in saponin production after elicitation

Elicitation of triterpene saponins is studied for the greater part in *Panax ginseng* (Table 1-2). MeJA in a concentration of 0.2 mM increased ginsenoside production 4 times in adventitious roots compared to the control (Ali et al. 2006) and 1.8 – 3.1 times in cell suspensions (Hu and Zhong 2007; Hu and Zhong 2008; Wang et al. 2005; Zhong and Zhang 2005). In response to 0.2 mM SA, 3 times more saponins were produced in *Panax ginseng* adventitious roots and HEJ increased ginsenoside production 2 – 4.4 times in cell suspensions (Ali et al. 2006; Hu and Zhong 2007; Hu and Zhong 2008). In *Glycyrrhiza glabra*, elicitation was tested in the roots of whole plants and it was found that 2 mM of MeJA and 1 mM of SA could increase saponin production 3.8 and 4.5 times, respectively (Table 1-2) (Shabani et al. 2009). Also for *Centella asiatica* whole plants were used and highest yields were reached with 0.01 mM MeJA in the leaves and 0.1 mM in the roots. Elicitation with 1 g/l yeast extract (YE) increased saponin production 1.4 times in whole plants of *Centella asiatica,* which was approximately the same with elicitation with 0.01 mM MeJA (Table 1-2) (Kim et al. 2004). For elicitation in cell cultures of the medicinal plant *Tabernaemontana catharinensis* different biotic elicitors were tested. Addition of cell wall homogenates to the cell cultures, no matter the concentration or type of microorganism source tested, resulted in increased triterpene levels. Among elicitors tested, *Saccharomyces cerevisiae* showed the best results in terms of triterpene accumulation (Pereira et al. 2007).

Table 1-2 Overview of successful elicitation strategies for triterpene saponins described in literature

Plant species	Culture system	Elicitor treatment			Saponin increase	Reference
		Elicitor	Concentration	Duration		
Panax ginseng	AR in bioreactor	MeJA	0.2 mM	7 d	**4.0 x**	(Ali et al. 2006)
		SA	0.2 mM	7 d	**3.0 x**	
Panax ginseng	AR	IBA	0.025 mM	10 d	**1.6 x**	(Zhong and Zhang 2005)
Panax notoginseng	CS	MeJA	0.2 mM	4 d	**3.0 x**	(Hu and Zhong 2008)
		HEJ	0.2 mM	4 d	**2.0 x**	
Panax notoginseng	CS in bioreactor	MeJA	0.2 mM	4 d	**2.6 x**	(Zhong and Zhang 2005)
Panax notoginseng	CS	MeJA	0.2 mM	15 d	**2.6 x**	(Wang et al. 2005)
	CS in bioreactor	MeJA	0.2 mM	15 d	**1.8 x**	
Panax notoginseng	CS	MeJA	0.2 mM	14 d	**3.1 x**	(Hu and Zhong 2007)
		HEJ	0.2 mM	14 d	**4.4 x**	
Medicago truncatula	CS	MeJA	0.5 mM	24 h	**10 x**	(Suzuki et al. 2002)
Glycyrrhiza glabra	Roots of whole plants	MeJA	2 mM	24 h	**3.8 x**	(Shabani et al. 2009)
		SA	1 mM	24 h	**4.5 x**	
Centella asiatica	Whole plants	MeJA	0.01 mM	7 d	**1.5 x**	(Kim et al. 2004)
		YE	0.1 g/l	7 d	**1.4 x**	
	Leaves of whole plants	MeJA	0.01 mM	36 d	**3.5 x**	
Centella asiatica	Aerial part of whole plants	MeJA	0.1 mM	35 d	**2 – 3 x**	(Mangas et al. 2006)
	Roots of whole plants	MeJA	0.1 mM	35 d	**4 – 6 x**	

MeJA = Methyl jasmonate, SA = Salicylic acid, IBA = Indole-3-butyric acid, HEJ = 2-hydroxyethyl jasmonate, YE = Yeast extract, AR = Adventitious roots, CS = Cell suspension

In general, it was also found that the elicitation of saponins was less effective in bioreactors compared to small-scale cultures. Wang et al. (2005) studied the difference in ginsenoside production in *Panax notoginseng* cell cultures in flasks and bioreactors. In flasks there was a sharp increase in total ginsenoside content from day 8 to day 15 after elicitation with a maximum on day 15, which was 2.6 times that of the control. In contrast, cells grown in bioreactors showed a slow increase in saponin production with a maximum on day 15 with 1.8 fold the concentration of the control. However, a repeated elicitation, 5 days after the first addition of MeJA, combined with sucrose feeding was suitable for the bioreactor cultivation of *P. notoginseng* cells for the hyper-production of ginsenosides (Wang et al. 2005).

Elicitation with MeJA or SA is often accompanied with a decrease in growth of the tissues. For *Panax ginseng* adventitious root cultures it was found that there was a severe decrease in biomass after

incubation for longer than 9 days in 0.2 mM MeJA or SA (Ali et al. 2006). Therefore phytohormones are sometimes simultaneously added with the elicitor. Kim et al. (2004) proved that addition of 0.025 mg/l thidiazuron (TDZ) could prevent the negative effects of MeJA on whole-plant growth and increased saponin production more than MeJA alone in whole plants. However, this increase was probably due to a gain in biomass rather than a stimulation of secondary metabolism. Because if only mature leaves were considered, there was no further increase in saponin content in addition to MeJA (Kim et al. 2004).
In some cases it is also reported that elicitor treatment of *in vitro cultures* not only increases the saponin production but additionally alters the stoichiometry of the precursors and final products. This is the case for example upon elicitation in large-scale cell cultures of *P. notoginseng* with 200µM MeJA or HEJ (Hu and Zhong 2007; Wang et al. 2005).

Upregulation of saponin biosynthesis genes in response to elicitation

Elicitation does not only have an effect on the saponin content but likewise also influences the expression of saponin biosynthesis genes. *Medicago truncatula* is a model species for plant functional genomics and produces at least five different triterpene aglycons. In addition, four putative early enzymes of triterpene biosynthesis were identified: squalene synthase (SS), β-amyrin synthase (βAS) and two squalene epoxidases (SE1 and SE2) (Suzuki et al. 2002). Therefore, *Medicago truncatula* is a good tool to study the behavior of saponin biosynthesis genes after elicitation. Suzuki and coworkers (2002, 2005) found that addition of abscisic acid (ABA) and SA to *Medicago* cell cultures had no effect on βAS, SS and SE transcript level; addition of yeast extract only had a week effect. In contrast, increasing concentrations of MeJA resulted in an accumulation of βAS transcripts, with a maximum of over 50 times increase, 24 hours after exposure to 500 µM MeJA. SS was coordinately induced. SE1 transcripts were not significantly induced but SE2 transcript induction closely followed βAS expression profile, indicating that SE2 but not SE1 may function specifically in the formation of triterpenoids (Suzuki et al. 2002; Suzuki et al. 2005).
Genetic studies were also performed in *Panax ginseng.* In this system, SS and SE transcription levels increased 9 to 6 times respectively, 24 hours after elicitation with 200 µM HEJ. Total ginsenoside increased from 12 hours to 10 days after addition of HEJ or MeJA. Upon addition of the jasmonate inhibitor DIECA (diethyldithiocarbamate), there was a decrease in both jasmonic acid production and ginsenoside content. DIECA also inhibited the HEJ induced upregulation of SS and SE, suggesting that induction of saponins is dependent on a jasmonate signal transduction (Hu and Zhong 2008).

In *Panax notoginseng* cell cultures, there was an increase in enzyme activity of UGRdGT, a glucosyltransferase that catalyzes the biotransformation from Rd1 into Rb1, two of the major saponins in *P. ginseng* (Wang et al. 2005). In contrast, ginsenoside-α-arabinofuranase, which hydrolyses ginsenoside Rc into Rd, was not detected. These results suggest that a biosynthesis pathway from Rd to Rb1 exists in the cell line and MeJA can activate this pathway by inducing UGRdGT activity (Wang et al. 2005). These results were confirmed by Hu & Zhong (2007), who found an increase in UGRdGT activity following elicitation with MeJA or HEJ and this coincided with a higher content of ginsenoside Rb1.

1.1.7 Metabolic engineering of triterpene saponin biosynthesis

Overexpression of key enzymes

Triterpenoid saponin backbones are synthesized via the isoprenoid pathway through a largely unidentified number of sequential enzymatic steps. Squalene is the precursor for triterpene saponins and is produced from mevalonic acid (MVA) through a series of enzymatic reactions with geranyl diphosphate synthase (GPS), farnesyl diphosphate synthase (FPS) and squalene synthase (SS) (Kuzuyama 2002) (Fig 1-1). Subsequently, squalene epoxidase transforms squalene into squalene-2,3-epoxide. The cyclization of squalene-2,3-epoxide into β-amyrin, α-amyrin and dammarenediol is catalyzed by squalene-2,3-epoxide cyclases (SECs) (Fig 1-2). More than 40 of these cyclases have been cloned from higher plants. Some of them have been functionally analyzed and characterized including β-amyrin synthase, α-amyrin synthase and dammarenediol-II synthase (Kim et al. 2009; Shibuya et al. 2009). The triterpenoid skeleton then undergoes various modifications, such as oxidation, substitution and glycosylation, also mediated by different enzymes. However, many of the enzymes involved in the later steps of saponin biosynthesis are not yet known or characterized. An increased production of triterpenoid saponins could be achieved by overexpression of key enzymes from the saponin biosynthesis pathway. A number of examples of metabolic engineering approaches are listed below.

Farnesyl diphosphate synthase (FPS)

Kim and coworkers (2010) investigated the role of FPS in triterpene biosynthesis in *Centella asiatica.* The plant was transformed with a construct harboring *Panax ginseng FPS* (PgFPS)-encoding cDNA coupled to the cauliflower mosaic virus 35S promoter (p35S). CaDDS (*C. asiatica* dammarenediol synthase) and CaCYS (*C. asiatica* cycloartenol synthase) mRNA showed high expression in all transgenic hairy root lines

when compared to the controls. The upregulation of CaDDS transcripts suggests that FPS may be an important enzyme in triterpene saponin production, which is reflected in enhanced saponin levels in transgenic lines (up to 1.5 times). Interestingly, the upregulated levels of CaCYS also correlated with increase in total sterol contents, which were 3 times higher than those of the controls. Therefore, these results indicated that overexpression of FPS is useful to enhance not only triterpene saponins, but also phytosterol production in plants (Kim et al. 2010).

Squalene synthase (SS)

The function of SS genes in the regulation of triterpene and phytosterol biosynthesis has been reported in several plants (Akamine et al. 2003; Devarenne et al. 2002; Hayashi et al. 1999; Kim et al. 2005; Lee et al. 2004; Seo et al. 2005; Uchida et al. 2009).

Overexpression of the *PgSS1* gene in adventitious roots of *P. ginseng* was followed by an upregulation of the downstream genes, such as squalene epoxidase, dammarenediol synthase, β-amyrin synthase and cycloartenol synthase (Lee et al. 2004). These results indicate that *PgSS1* is a key regulatory enzyme for both phytosterol and triterpenoid saponin biosynthesis. The *PgSS1* gene derived from *P. ginseng* has also been introduced into Siberian ginseng (*Eleutheroccous senticosus*) (Seo et al. 2005). The transgenic *E. senticosus* plant showed enhanced production of phytosterol (β-sitosterol and stigmasterol). In addition, triterpene saponin (ciwujianosides B, C_1, C_2, C_3, C_4, D_1 and D_7) levels also increased by 2 to 2.5 fold.

Squalene epoxidase (SE)

Squalene epoxidase (SE), also referred to as squalene monooxygenase, catalyzes the first oxygenation step in phytosterol and triterpenoid saponin pathways. Plants, so far examined, seem to have two or more copies of SE genes. In *Arabidopsis*, six isoforms of SE have been identified, suggesting that SE genes have different isoform-dependent functions (Rasbery et al. 2007).

The role of two squalene epoxidase genes (*PgSE1* and *PgSE2*) has been studied in *P. ginseng* (Han et al. 2010). Amino acid sequences deduced from *PgSE1 and PgSE2* shared 80% homology, but the N-terminal region (first 60 amino acids) was highly divergent. *PgSE1* was abundantly accumulated in all organs, while *PgSE2* was weakly expressed in petiole and flower buds. RNAi of *PgSE1* in transgenic *P. ginseng* completely suppressed *PgSE1* expression, thus reducing ginsenoside production. Interestingly, silencing of *PgSE1* strongly upregulated *PgSE2* and cycloartenol synthase, which resulted in enhanced phytosterol accumulation. These results indicate that expressions of *PgSE1* and *PgSE2* are regulated differently.

Furthermore, *PgSE1* only regulates ginsenoside, and not phytosterol biosynthesis. Therefore, overexpression of *PgSE1* could be useful to increase the production of ginsenoside in *P. ginseng*.

β-Amyrin synthase (βAS)

The most studied triterpenes found in higher plants are those from the oleanane type (β-amyrin), followed by ursane (α-amyrin) and dammarane (dammaranediol) types (Shibuya et al. 2009). In addition, it is already well known that the first committed step in saponin biosynthesis in plant involves the cyclization of squalene-2,3-epoxide into the saponin types mentioned above. These conversions are catalyzed by specific squalene-2,3-epoxide cyclases (SECs) including β-amyrin synthase. The genes encoding β-amyrin synthase have been cloned and characterized from different plant species such as *Panax ginseng* (Kushiro et al. 1998), *Pisum sativum* (Morita et al. 2000), *Glycyrrhiza glabra* (Hayashi et al. 2001), *Avena strigosa* (Haralampidis et al. 2001), *Medicago truncatula* and *Lotus japonicus* (Iturbe-Ormaetxe et al. 2003) and many other plants (Cammareri et al. 2008; Kajikawa et al. 2005; Liu et al. 2009; Meesapyodsuk et al. 2007; Scholz et al. 2009; Shibuya et al. 2009; Zhang et al. 2003).

The effect of ectopic expression of *AsOXA1* cDNA, a β-amyrin synthase from *Aster sedifolius,* has been evaluated for the production of saponins in *Medicago truncatula* (Confalonieri et al. 2009). One of the four transgenic lines expressing *AsOXA1* significantly accumulated larger amounts of triterpenoid compounds in leaves and roots compared to that in control plants. The increase in the total amounts of triterpenoid saponin observed in leaves of transgenic lines correlated with the *AsOXA1* expression level. Furthermore, the plants expressing *AsOXA1* not only produced higher level of saponin, but also improved root nodulation. It is suggested that the high-nodulating phenotype could be associated with the significant increase in saponin production in the root. A similar effect has also been reported in *Glycyrrhiza glabra* (Hayashi et al. 2004).

β-Amyrin synthase is one of SECs located at the branch point for sterol (primary metabolism) and triterpene saponin (secondary metabolism) biosynthesis, therefore knowledge on the regulation of β-amyrin synthase will be critical to understand the regulation, mechanism and physiology of secondary metabolism. So far there is one report (Confalonieri et al. 2009) on the manipulation of the triterpene saponin pathway based on the expression of β-amyrin synthase encoding gene. Since the specific product of β-amyrin synthase is one of the key precursors of triterpene saponin pathway, overexpression of β-amyrin synthase is likely to yield an increased saponin production.

Cytochrome P450 monooxygenases (P450s)

The aglycons of triterpene saponins are C30 compounds produced by the cyclization of squalene-2,3-epoxide. Subsequent oxidations of the triterpene skeleton produce structural diversity and these oxidations are thought to be catalyzed by cytochrome P450 monooxygenases (P450s). Numerous P450s have been suggested to be involved in the biosynthesis and metabolism of triterpenoid saponins (Ohnishi et al. 2009; Seki et al. 2008; Shibuya et al. 2006).

Several studies have been performed to elucidate the avenacin pathway, triterpene saponins produced by oats (*Avena* spp.). Genetic analysis of *saponin-deficient* (*sad*) mutants revealed nine loci involved in avenacin biosynthesis, eight of which are clustered and only one, *Sad 4*, that is unlinked (Mylona et al. 2008). Qi and coworkers (2006) have investigated the *sad2* mutant and showed that it is a cytochrome P450, designated CYP51H10. The CYP51H10 enzyme catalyzes the conversion of β-amyrin to avenacin and its expression is restricted to the root epidermis (Qi et al. 2006). This result correlated with the synthesis of avenacin A-1, which is tightly regulated and is restricted to the epidermal cell layer of root tips. However, the catalytic function of CYP51H10 has not been clearly characterized. The CYP51H10 belongs to the CYP51 sterol demethylase family. This enzyme is highly conserved in the synthesis of essential sterols, only found in monocots and is regarded as the most ancient cytochrome P450 family. These data indicated that, in oats, CYP51H10 has been evolved from CYP51 family to adapt to triterpene molecules.

In *P. ginseng*, ginsenosides are presumably synthesized from dammarenediol II after hydroxylation by cytochrome P450 (Shibuya et al. 2006). Cytochrome P450 members in *P. ginseng* are suggested to be involved in the hydroxylation of the C-12 position of dammarenediol for protopanaxadiol synthesis and the C-6 position of protopanaxadiol for protopanaxatriol synthesis. Both of these compounds are used as backbones for ginsenosides.

The advancement of DNA sequencing techniques has generated a great opportunity to identify a multitude of candidate P450 gene sequences. The genomic studies of model plant system provide a future basis for discovering novel P450 functions. In comparison to the availability of sequence information, only limited biochemical characterization has been achieved so far (Mizutani and Ohta 2010; Ohnishi et al. 2009). Eventually, the objective of these P450s studies should be a global annotation that describes the enzymatic activity of each gene.

Glycosyltransferases (GTs)

Glycosylation generates more structural diversity of triterpene saponins in a plant than do cyclization and oxidation (Phillips et al. 2006; Xu et al. 2004). However, identification of sugar transferases involved in saponin biosynthesis remains a problem due to the large number of candidate genes and the structural complexity of the sugar chain present in triterpene saponins.

Recently, six glycosyltransferases (GTs) have been functionally characterized: UGT71G1, UGT73K1 and UGT73F3 from *M. truncatula* (Achnine et al. 2005; Naoumkina et al. 2010), UGT74M1 from *S. vaccaria* (Meesapyodsuk et al. 2007) and UGT73P2 and UGT91H4 from *G. max* (Shibuya et al. 2010). In *M.* truncatula, UGT71G1 has specificity for medicagenic acid and also some flavonoids, while UGT73K1 specifically has shown glucosyltransferase activity for hederagenin and soyasapogenol B and E. Genetic loss of function analysis of UGT73F3 has indicated that glucoside produced by the action of UGT73F3 on hederagenin as sugar acceptor is a C-28 ester. In addition, analysis of an expressed sequence tags (EST) library of *S. vaccaria* revealed that UGT74M1 is a triterpene carboxylic acid glucosyltransferase. UGT74M1 is expressed in roots and leaves and appears to be involved in vaccaroside biosynthesis. UGT71G1, UGT73K1, UGT73F3 and UGT74M1 are primary GTs that transfer one sugar molecule to a triterpene aglycon to yield a triterpene saponin bearing a monosaccharide.

Further study was conducted on *G. max* to unravel how the sugar moiety of saponins with two or more saccharides is configured (Shibuya et al. 2010). Identification of UGT73P2 and UGT91H4 showed that these GTs were involved in soyasaponin biosynthesis and they could yield triterpene saponins bearing a dissacharide and trisaccharide, respectively. Remarkably, this is the first report of GTs that transfer the second and third sugar to the triterpene saponin skeleton. The results also indicate that the sugar chain moiety of triterpene saponins is synthesized by successive sugar transfer reactions to aglycons.

1.2 *Maesa* species

1.2.1 Taxonomy, morphology and occurrence

Maesa is a genus that comprises approximately 100 species, from small trees and scandent shrubs to woody vines from rainforest canopies. They occur primarily in the tropics of Africa and Asia. *Maesa* spp. have been placed in the family of Myrsinaceae (order of Ericales), stressing however the differences with other members in the family, especially in floral characteristics (Caris et al. 2000). Because of these differences, Anderberg suggested in 1995 that the genus *Maesa* should be removed from the Myrsinaceae and placed in a newly defined family, Maesaceae, more closely related to Primulaceae (Anderberg and Stahl 1995). Today there is still some confusion and *Maesa* spp. are classified as Myrsinaceae by some authors and as Maesaceae by other authors.

Generally, *Maesa* plants flower early in the year, however, the flowering period is strongly affected by climate conditions. A thorough investigation of *M. perlarius* and *M. japonica* flowers showed that both species are functionally dioecious. This means that the species have two sexual phenotypes differing in both primary and secondary sexual characteristics. The consequence is that there are plants that will set fruit (with a functional pistillate) and plants that will not set fruit (with a functional staminate) (Utteridge and Saunders 2001). Functional dioecy has also been documented in 4 Philippine *Maesa* species (Utteridge and Saunders 2004), therefore it appears likely that many *Maesa* spp. will show a degree of sexual dimorphism with associated functional dioecy.

For this research four *Maesa* species were used (Fig 1-3). *Maesa argentea* (Fig 1-3a) is a shrub but can grow up to 5m tall in broad-leaved forests, hilly areas, valleys, stream banks and damp places. They are found in many Asian countries and in the complete Himalaya region. *Maesa balansae* (Fig 1-3b) is mainly found in Vietnam and China. *Maesa perlarius* (Fig 1-3c) is a small shrub of 1 to 3m tall and is found in broad-leaved forest, shrubby areas, hillsides and damp places in Thailand, Vietnam and China. *Maesa lanceolata* (Fig 1-3d) occurs primarily in central and south-east Africa (e.g. in Rwanda, Kenya, Madagascar, Tanzania, South-Africa and Zimbabwe), however, the habitat has also spread to Saudi Arabia and India. They can be straggling shrubs of 2 to 3m tall or small trees up to 9m. They grow also on cliff tops as in midland or coastal areas.

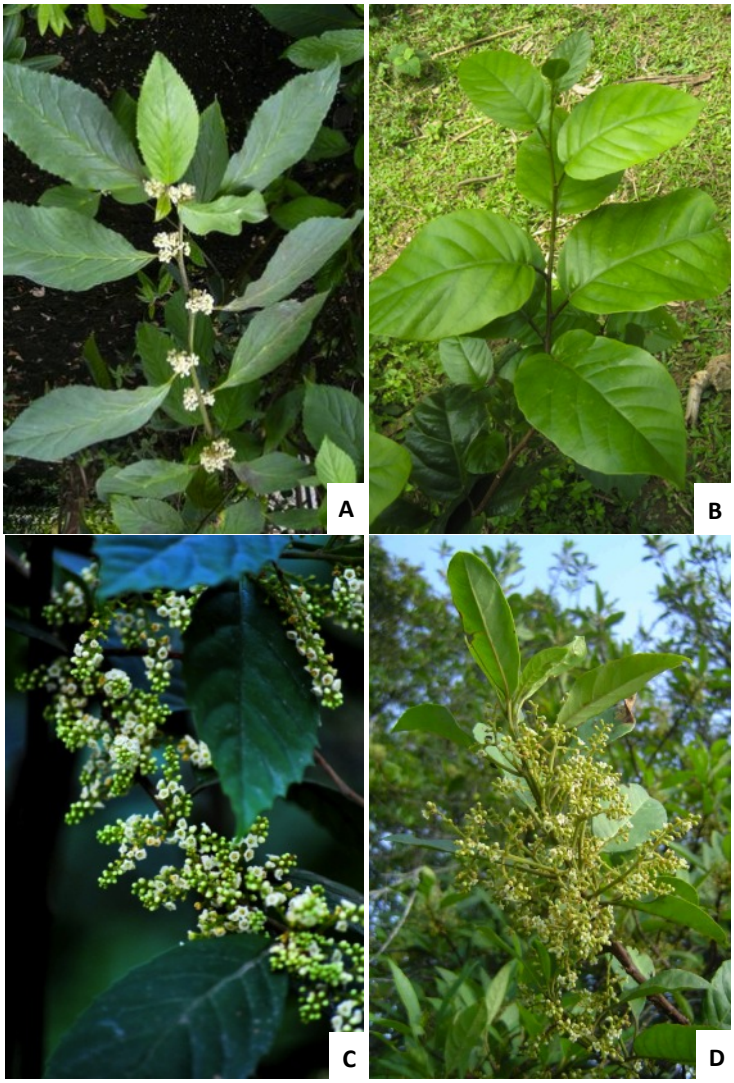


Fig 1-3 Pictures of *Maesa argentea* (**a**), *Maesa balansae* (**b**), *Maesa perlarius* (**c**) and *Maesa lanceolata* (**d**).

1.2.2 Saponin production in *Maesa* species

All four *Maesa* species studied in this work have been referred to traditional medicine and ancient practices. For instance, a paste of *Maesa perlarius* leaves is used to heal broken bones in China. In Cambodia, Laos and Vietnam, the roots of *Maesa perlarius* are used to promote digestion and urination and an extract of the leaves is used to cure measles (Wiart 2006). *Maesa balansae* is used for the treatment of allergies, sprains, helminthic infections, drunkenness and headache in the Northern part of Vietnam (Germonprez et al. 2004). The fruits of *Maesa argentea* are eaten and squeezed leaves are used as a fish poison in Nepal (Wiart 2006). *Maesa lanceolata* is well known in Rwandan traditional medicine, where the extracts of leaves and fruits are used against various diseases including hepatitis, dysentery,

skin diseases and neuropathies (Sindambiwe et al. 1996). *Maesa lanceolata* is also used in the Eastern part of Africa for prevention of cholera (Okemo et al. 2003). Detailed studies on the constitution of these plants extracts and *in vitro* and *in vivo* bioactivity assays showed that mainly triterpene saponins are responsible for this broad spectrum of biological actions.

Maesa lanceolata: 'maesasaponins'

The first report on triterpenoid saponins in *Maesa lanceolata* was published in 1996 by Sindambiwe and co-workers. Within the framework of research on natural anti-infectious agents, investigations were conducted on *Maesa lanceolata*. Using a bioassay-guided fractionation, a saponin mixture was isolated from the leaves of *M. lanceolata*. Within the mixture, six homologous triterpene saponins were identified using nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) (Sindambiwe et al. 1996). 2 years later an effort was undertaken to obtain pure saponins via a semipreparative high performance liquid chromatography (HPLC) method. In this way 14 closely related oleanane-type saponins were obtained (Apers et al. 1998). Subsequently, a study was performed to elucidate the structure of these new acylated saponins and 10 different triterpene saponins were identified. The saponins constituted a structurally consistent series of mono-, di- and triesters and were named **maesasaponins** (Apers et al. 1999). Further investigations using HPLC-MS lead to the characterization of 5 more triterpene saponins compounds; however, these structures are not yet confirmed with NMR (Fig 1-4) (Theunis et al. 2007).

	R1	R2	R3
Maesasaponin I	H	angeloyl	H
Maesasaponin II	acetyl	angeloyl	H
Maesasaponin III$_1$*			
Maesasaponin III$_2$	H	angeloyl	acetyl
Maesasaponin IV$_1$*			
Maesasaponin IV$_2$	acetyl	angeloyl	acetyl
Maesasaponin IV$_3$	H	angeloyl	propanoyl
Maesasaponin V$_1$*			
Maesasaponin V$_2$	acetyl	angeloyl	propanoyl
Maesasaponin V$_3$	H	angeloyl	butanoyl
Maesasaponin VI$_1$*			
Maesasaponin VI$_2$	H	angeloyl	angeloyl
Maesasaponin VI$_3$	acetyl	angeloyl	butanoyl
Maesasaponin VII$_1$	acetyl	angeloyl	angeloyl
Maesasaponin VII$_2$*			

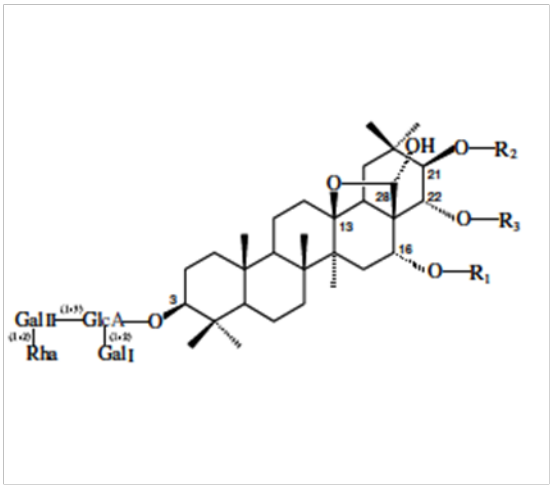


Fig 1-4 Structures of the known saponins in *Maesa lanceolata* leaves. *Structures are not yet identified but compounds are characterized based on their molecular mass (figure adapted from Theunis et al. (2007)).

Maesasaponins proved to have many interesting biological actions, however, up to date mainly *in vitro* tests were performed because of the cytotoxic and haemolytic side effects of the saponins. The most important biological activities of maesasaponins are summarized below. In addition, some efforts to establish structure – activity relationships are mentioned.

- The maesasaponin mixture showed moderate **virucidal activity** against all envelope viruses tested (herpes simplex virus type 1, poliovirus, vesicular stomatitis virus T2, semliki forest virus A7 and measles virus strain Edmonston A) (Sindambiwe et al. 1999; Sindambiwe et al. 1998). When the saponins separately were tested for virucidal actions, it seemed that maesasaponin V_3 was the most active virucidal compound. Maesasaponins III_2, IV_3 and VI_2 had a moderate activity against viruses, the remaining saponins only showed a weak activity. So it appears that only 21,22-diesters have a virucidal effect (Apers et al. 2001).
- Reports on the **fungicidal activity** of maesasaponins are somewhat contradictory. A test performed by Sindambiwe and coworkers (1998) with a saponin extract from *M. lanceolata* leaves showed **fungistatic** but not fungicidal activity against 6 fungi. Okemo and coworkers (2003) tested 10 other fungi with a methanolic extract from *M. lanceolata* bark. The saponin mixture showed strong antifungal activity against 8 fungi and moderate activity with 2 fungi (Okemo et al. 2003). Probably the saponin mixtures in *M. lanceolata* leaves and bark differ, which could lead to different biological activities.
- The aquatic snail *Biomphalaria glabrata* is an intermediate host for trematodes of the genus *Schistosoma*, causing the chronic parasitic disease schistosomiasis. Molluscicidal agents may play an important role in the prevention of the disease. The maesasaponin mixture showed very strong **molluscicidal activity** against *B. glabrata* (Sindambiwe et al. 1998). Of the individual saponins, only maesasaponin VI_2 showed higher activity than that of the mixture with a 50% lethal dose at 2.3µg/ml (Apers et al. 2001). So *M. lanceolata* may be a real candidate for snail control programs, however, saponins also have an effect on other aquatic biota. A 10 minutes contact is sufficient to kill fish with the crude saponin extract (lethal dose = 3µg/ml). Also larvae from mosquitoes are very sensitive to the saponin extract (lethal dose = 1µg/ml) (Bagalwa and Chifundera 2007). Consequently, attention must be paid when using *M. lanceolata* extracts to control snails to avoid ecological disturbances in the environment.
- Angiogenesis is the development of new blood vessels from an established microvasculature, which is an important process in many diseases such as neovascular glaucoma, benign and malignant tumors. Inhibition of angiogenesis could therefore be a novel therapeutic strategy for

a number of diseases. **Antiangiogenic activity** can be assessed using different *in vitro* and *in vivo* tests. A commonly used *in vivo* model, chick chorioallantoic membrane (CAM) assay[1], was used to determine the antiangiogenic activity of maesasaponins. The saponin mixture showed moderate antiangiogenic activity and inhibited angiogenesis in a dose dependent manner within a concentration range of 2.5 to 10 µg/pellet. Little membrane damage was also noticed with concentrations lower than 25 µg/pellet. At a concentration of 10 µg/pellet, the individual saponins all showed good inhibition of angiogenesis, however, at a concentration of 1 µg/pellet different effects were noticed. At this low concentration maesasaponin IV_2 and VI_3 were not active, maesasaponins I, IV_3, V_2 and VII_1 had a weak activity and maesasaponins II, V_3 and VI_2 showed very strong activity. Maesasaponin II was found to be the most active saponin in the antiangiogenesis assay. In addition, maesasaponin II showed the weakest membrane damage (Apers et al. 2002).

- One of the major drawbacks of maesasaponins is the very high **haemolytic activity**: the 50% haemolyzing concentration of the saponin mixture was only 1.6µg/ml (Sindambiwe et al. 1998). Individual saponins were also tested for haemolytic activity and 21,22-diesters were found to be the most active compounds (maesasaponins IV_3, V_3 and VI_2). The corresponding triesters were less active, so it was suggested that a free 16 OH group is correlated with a high haemolytic activity. Maesasaponin I and II caused no haemolysis, not even at the highest concentration tested (20µg/ml); therefore, a substitution at position 22 is probably also required for a high haemolytic activity (Apers et al. 2001).
- Another side effect of maesasaponins is a very high **cytotoxicity** with a 50% cytotoxicity concentration of less than 15µg/ml for the saponin mixture and 21,22-diesters (more specifically maesasaponins III, IV_3, V_3 and VI_2) (Apers et al. 2001).

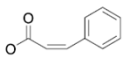
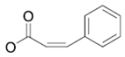
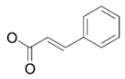
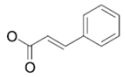
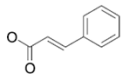
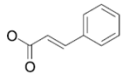
Likewise, the aglycon mixture was tested for biological activities but had very low activity compared to the normal saponin mixture. So it seems that glycosylation at position C3 is essential for the biological activities of the maesasaponins (Sindambiwe et al. 1998). Many of the observed activities are probably caused by interaction of the saponins with cell membrane components or, in the case of viruses, through interaction with viral envelope components (Sindambiwe et al. 1998).

[1] Vascular damage of the chorioallantoic membrane of a 10 days old fertilized chick egg is measured following exposure to test chemicals.

Of all *Maesa lanceolata* saponins, **maesasaponin II** seems to be the most promising one. It has the strongest antiangiogenic activity with the weakest membrane damage (Apers et al. 2002). In addition, maesasaponin II scores best in haemolytic tests; with no haemolytic activity, not even at the highest concentration tested (20µg/ml) (Apers et al. 2001).

Maesa balansae: 'maesabalides'

During a random drug screening program, the methanolic extract of *Maesa balansae* leaves was found to possess very strong antileishmanial activity. Six related triterpenoid saponins were identified as the active constituents. The structures of these saponins were determined and are shown in Figure 1-5 (Germonprez et al. 2004). *M. balansae* saponins were named **maesabalides**.

Compound	R^1	R^2	R^3
1			OH
2			OH
3			OH
4			OH
5			OAc
6			OAc

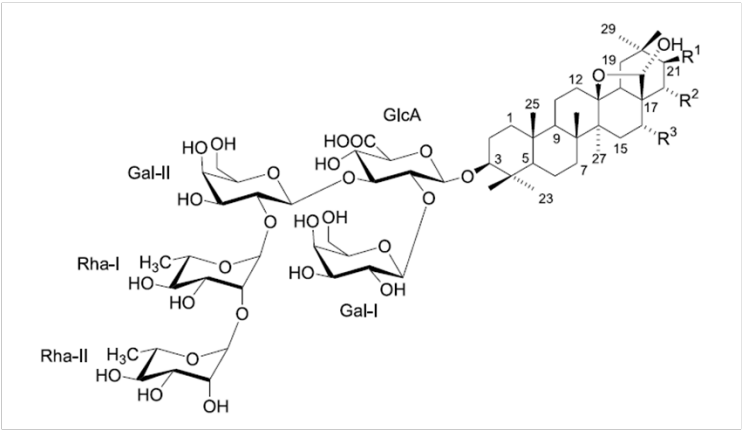


Fig 1-5 Structures of the saponins in *Maesa balansae* leaf extracts (figure adapted from Germonprez et al. (2004))

Since the initial tests showed that *Maesa balansae* extracts were very promising for the treatment of leishmaniasis, the majority of the biological tests focused on **antileishmanial action**. Leishmaniasis is a parasitic protozoal disease, occurring mainly in tropical and subtropical areas. The disease is a growing health problem, with 12 million patients over the world and approximately 2 million new cases every year. Treatment options are currently limited and rely on pentavalent antimonials as first-line and amphotericin B and pentamidine as second-line drugs. Both the emergence of resistance to the first-line drugs and the excessively high cost of amphotericin B explain why new drug discovery must remain a primary objective (Croft et al. 2006; Maes et al. 2004a; Maes et al. 2004; Maes et al. 2004b; Vermeersch et al. 2009).

In vitro tests, using the strain *Leishmania donovani,* showed that the maesabalides mixture was very active with a 50% inhibitory concentration (IC_{50}) of 0.4µg/ml. This was comparable to the IC_{50} of amphotericin B. In addition, no cytotoxicity was noticed on the host cells. The same mixture was also effective (without side effects) in small scale *in vivo* tests with BALB/c mice infected with *L. donovani* (Maes et al. 2004b). Further *in vivo* studies were conducted using hamsters as a model system instead of mice. These experiments confirmed that the maesabalides mixture was well tolerated and could result in full recovery from clinical disease. When testing the individual saponins in the mixture, maesabalides III was the most active one. Like the mixture, maesabalides III was able to cure the clinical symptoms with a potency that was comparable to amphotericin B (Maes et al. 2004a).

Maesa argentea and Maesa perlarius

Presence of saponins in *Maesa argentea* and *M. perlarius* was first confirmed by Foubert and coworkers in 2008. Some of the saponins were identified through comparison of the molecular weight, MS fragmentation pattern and retention time with those of reference samples of maesasaponins and maesabalides (Table 1-3). Based on these data it could be concluded that both twigs and leaves of *M. argentea* contained maesasaponin III_2, IV_3, V_3 and VI_2. In addition, *M. argentea* leaves showed two new peaks in the chromatogram. *M. perlarius* contained products with the same molecular weight as the maesasaponins and maesabalides, however, with differences in retention time and/or MS fragmentations pattern (Foubert et al. 2008). This study already gives an idea about the saponins in *M. argentea* and *M. perlarius*, though, more in depth investigations are necessary to obtain conclusive data on the saponins produced in these two species.

Table 1-3 Comparison of LC-MS data from *Maesa argentea* twigs and leaves and *Maesa perlarius* twigs with known data from *Maesa lanceolata* saponins (maesasaponins) and *Maesa balansae* saponins (maesabalides). Table adapted from Foubert et al. 2008.

Saponins		*M. argentea twigs*	*M. argentea leaves*	*M. perlarius twigs*
Maesasaponin	I	-	X	-
	II	-	-	-
	III_1	-	-	-
	III_2	X	X	-
	IV_1	-	-	-
	IV_2	-	-	-
	IV_3	X	X	-
	V_1	-	-	-
	V_2	-	-	-
	V_3	X	X	-
	V_4	-	-	-
	VI_1	RT	-	-
	VI_2	X	X	-
	VI_3	-	-	-
	VII_1	-	-	-
	VII_2	-	-	-
Maesabalides	I	-	-	Y
	II	-	-	-
	III	-	-	-
	IV	-	-	RT
	V	-	-	RT
	VI	-	-	RT

X = identical retention time and MS fragmentation pattern, RT = identical retention time, MS = identical fragmentation pattern, Y = identical molecular weight

Also concerning biological actions these two species have been less thoroughly studied compared to *M. lanceolata* and *M. balansae.* A butanol extract of *M. argentea* and *M. perlarius* leaves and twigs showed strong anti-leishmanial action in an *in vitro* test with *L. infantum* (IC_{50} < 0.125 µg/ml). However, this was accompanied with a serious cytotoxicity (IC_{50} = 1.85 – 6.86 µg/ml). The ethanol extract of *M. perlarius* twigs also showed high anti-leishmanial activity (IC_{50} = 1.75 µg/ml) but with lower cytotoxicity (IC_{50} = 39 µg/ml) (Foubert et al. 2008). A subsequent study using the same tests confirmed the very strong anti-leishmanial activity of a methanol extract of *M. argentea* leaves against *L. infantum* (IC_{50} < 0.125 µg/ml). Though, cytotoxicity is about 10 times lower (IC_{50} = 18 compared to 1.85 µg/ml in previous tests). For *M. perlarius* only the ethanol extract was investigated with the same results as previously reported (Vermeersch et al. 2009).

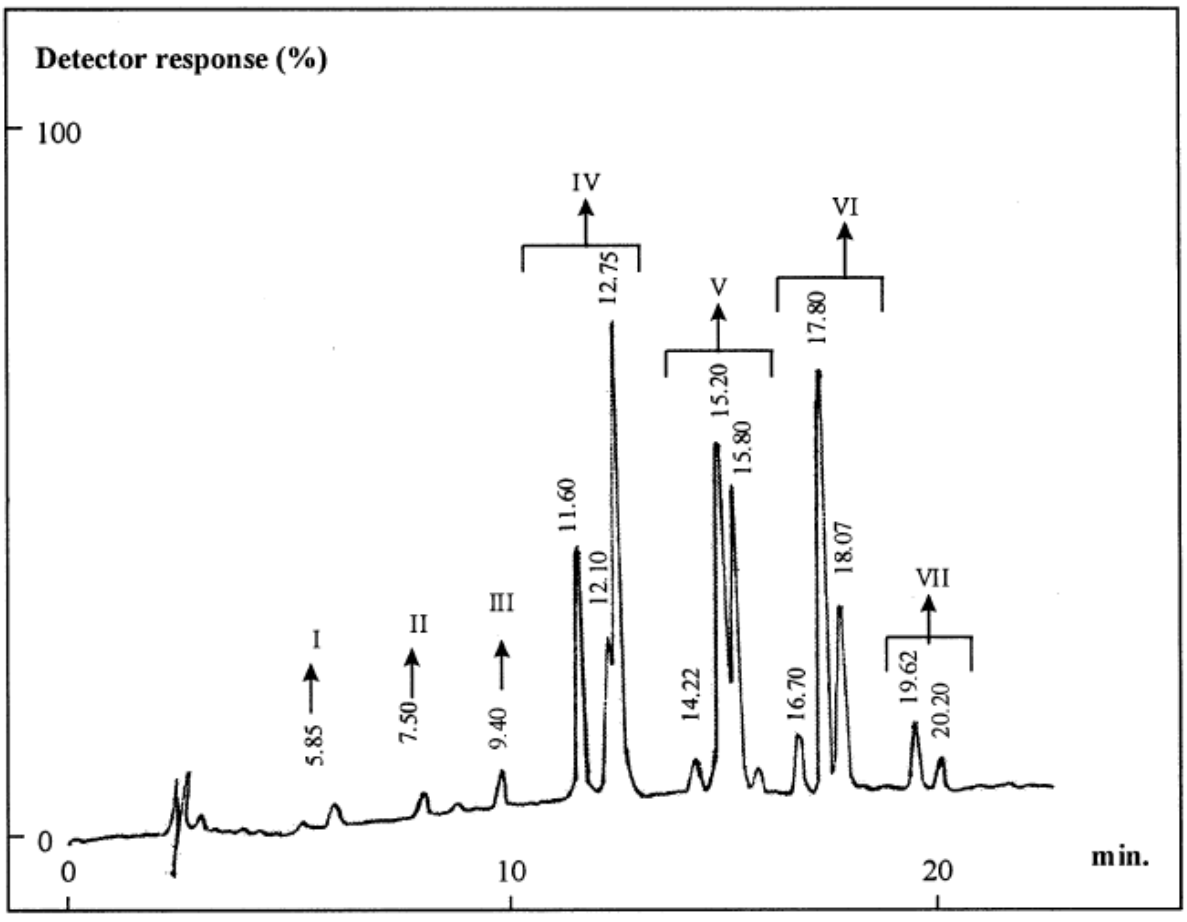
Other secondary metabolites in Maesa species

A screen of East-African plants for antimicrobial activity in 1978 revealed that *Maesa lanceolata* had a strong antibacterial activity. Further investigations led to the isolation of a benzoquinone, which was called **maesanin**, as the active compound (Taniguchi et al. 1978). Further studies confirmed the *in vivo*

and *in vitro* antibacterial and antifungal activity of maesanin (Kubo et al. 1983). In more recent studies, the isolation and structure determination of more benzoquinone derivatives from *M. lanceolata* fruits is described (Manguro et al. 2003; Mossa et al. 1999). Evaluation of *in vitro* cytotoxic and antioxidant activities of natural and semi-synthetic benzoquinones from *M. lanceolata* fruits showed that the biological properties of the benzoquinones were strongly dependent on their structure. Some benzoquinones show a strong cytotoxic effect against different types of cancer cell lines, other structures show a prominent antioxidant effect (Muhammad et al. 2003). There are no studies available on the combined effect of benzoquinones and saponins in *Maesa lanceolata*.

To the best of our knowledge, no bioactive secondary metabolites, other than saponins, are described for *M. argentea, M. balansae* and *M. perlarius*.

CHAPTER 2 SAPONIN PRODUCTION IN MAESA PLANTS



2.1 Abstract

Saponins are present in many plants species and the content of saponins can depend on a multitude of factors such as cultivar, age, physiological state, geographical location and environmental conditions. Even within one plant there can be a large variation in saponin content between different organs or tissues. In this chapter, a saponin extraction and thin layer chromatography (TLC) method were fine-tuned for rapid and efficient study of *Maesa* saponins. TLC analyses of greenhouse plants revealed that saponin content is different for different tissues. In addition, we found that younger plant material contained less saponins than older plants or leaves. Treatment of *ex vitro* plants with methyl jasmonate or saponin precursors did not affect saponin production. Based on these observations we conclude that saponins in *Maesa* species can be assigned to the class of phytoanticipins (constitutive phytoprotectants) rather than phytoalexins (inducible phytoprotectants).

2.2 Introduction

Saponins occur in a wide variety of plant species, both in wild plants and cultivated crops. Triterpenoids are found principally in dicotyledonous species, while many of the major steroidal saponins are synthesized in monocots (Osbourn 2003). Despite the sometimes negative biological effects of the saponins on animals and humans, they do occur in many crops and edible plants. Examples are soybean (Berhow et al. 2006), tea (Kohata et al. 2004), leek (Carotenuto et al. 1999), onion, garlic (Lanzotti 2006), cayenne pepper (De Lucca et al. 2006), pea (Taylor et al. 2004), tomato (Fujiwara et al. 2003) and leguminous forage species such as alfalfa (Oleszek 1998). Saponins are also present in numerous medicinal plants and herbs used in traditional medicine; for example *Centella asiatica* (James and Dubery 2009), *Panax ginseng* (Wu and Zhong 1999), *Glycyrrhiza glabra* (Shabani et al. 2009), *Bupleurum falcatum* (Kim and Park 2001) and *Quillaja saponaria* (Bankefors et al. 2008). The content of saponins depends on many factors such as cultivar, age, physiological state and geographical location of the plants. In addition, the distribution within different organs of a plant varies considerably (Hostettmann and Marston 1995). The role of saponins is not yet completely elucidated, though, it is generally accepted that they have a role as phytoprotectants. These types of molecules are either produced upon a stimulus conveyed by the pathogen or produced in a developmentally controlled fashion. Inducible phytoprotectants are known as **phytoalexins**, while constitutive phytoprotectants are called **phytoanticipins**.

Many different methods have been used for the qualitative and quantitative determination of saponins. They can be divided in two groups: non-chromatographic and chromatographic techniques. Non-chromatographic methods are based on haemolysis, piscicidal activity, gravimetry, spectrophotometry or immunoassays with monoclonal antibodies. Examples of chromatographic methods are thin layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC), ultra performance liquid chromatography (UPLC) and so on (Hostettmann and Marston 1995; Oleszek and Bialy 2006). For the analysis of *Maesa* saponins three different techniques can be used; **TLC** for fast detection of saponins and relative differences between samples, **HPLC** in combination with mass spectrometry (**MS**) for quantitative saponin measurements and nuclear magnetic resonance (**NMR**) analysis for identification and characterization of (new) saponin structures.

In this chapter, we have fine-tuned a protocol for saponin extraction from *Maesa* species that allowed rapid and efficient extraction in small volumes. In addition, thin layer chromatography (TLC) is suggested as a tool for investigation of saponin production in *Maesa.* TLC is used as a supporting technique for rapid analysis of saponin fractions. The major advantage is that TLC does not require specialized equipment. In addition, it is rapid, relatively inexpensive and the TLC plates can handle both pure saponins and crude extracts (Hostettmann and Marston 1995). Using these methods, we have examined saponin production in four species under different developmental and environmental conditions.

2.3 Results

2.3.1 Crude saponin extraction for TLC analysis using a miniaturized protocol

Methods for crude saponin extraction from *M. lanceolata* leaves were already described in several research articles (Apers et al. 1998; Sindambiwe et al. 1996; Theunis et al. 2007). However, we experienced two major disadvantages using these methods: a large amount of starting material was needed (500mg) and the extraction procedure took a long time because only one sample could be handled at a time. The most time-delaying step was drying the samples under vacuum; 10ml of a 50% methanol extract needed to be dried using a rotary evaporator (Rotavap), this took more or less four hours for one sample. Therefore, we adjusted the protocol in such a way that we could work in Eppendorfs and that we needed only 50mg of starting material. This was brought into 250µl of 50% methanol and sonicated for 1 hour. The samples were then centrifuged for 10 min (2000 rpm), the supernatant was brought into a new Eppendorf and the pellet was dissolved in 250µl 50% methanol and

again sonicated for 1 hour. In the end, both supernatant fractions were combined and dried using a HetoVac centrifuge during 4 hours. In this way, 60 samples could be handled in one time, which significantly increased the efficiency of the extraction method. Saponin extracts could subsequently be analyzed using thin layer chromatography (TLC).

2.3.2 Saponin studies in *Maesa* greenhouse grown plants

Saponin content in Maesa leaves, stems and roots

To analyze the presence of saponins in different organs, crude extracts were made from leaves, stems and roots of *Maesa argentea, M. balansae, M. lanceolata* and *M. perlarius*. These were subjected to thin layer chromatography (Fig 2-1).

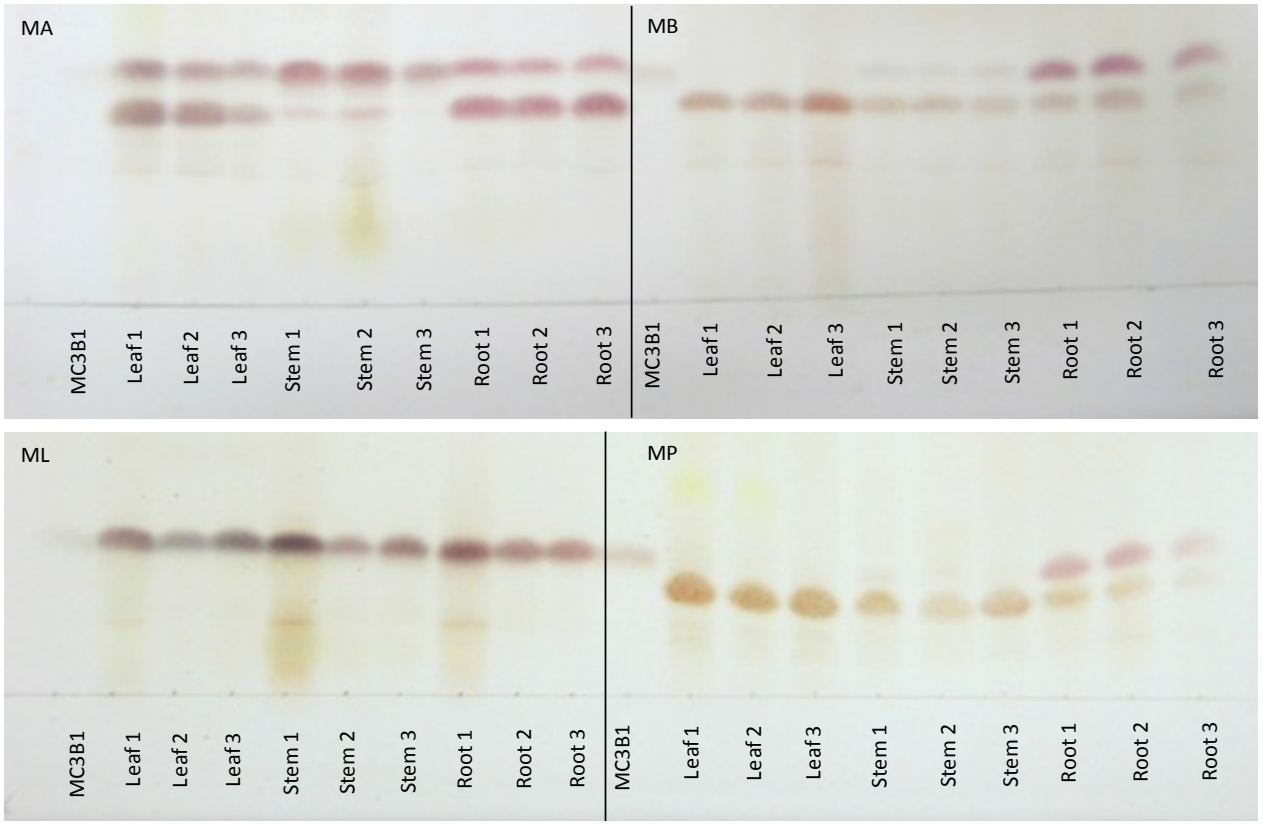


Fig 2-1 TLC analysis of leaves, stems and roots of *M. argentea* (MA), *M. balansae* (MB), *M. lanceolata* (ML) and *M. perlarius* (MP). For each species, three different plants were used for extraction. A HPLC purified maesasaponin mix (MC3B1) was used as reference sample.

For *M. argentea* two bands were visible in all organs; the lower band, with retention factor (R_f) 0.17, being the most abundant in leaves and roots. In stems, the upper fraction with R_f 0.14 was clearly more abundant. In *M. balansae* shoots only one band was observed with R_f 0.16, in stems a small upper band with R_f 0.14 also appeared. In roots both fractions were present and had more or less the same size, however, the color slightly differed. *M. lanceolata* had one band with R_f 0.17 in all organs. Finally, in *M. perlarius* a similar pattern was found as in *M. balansae*; one lower band with R_f 0.16 in leaves, a very small upper band with R_f 0.14 appearing in the stems and finally two bands of the same size in the roots. For all species, the upper bands were most likely *Maesa* saponins as they were very similar to the MC3B1 control. MC3B1 is the pure maesasaponin mixture from *M. lanceolata* leaves and was used as a reference sample for TLC. The lower bands were probably also saponins, based on their color and shape although, for *M. balansae* and *M. perlarius* the colors of the two bands were slightly different. This could indicate a small difference in the aglycon structure or in the sugar moiety attached to it. We used a normal phase TLC plate and therefore we can only conclude that the lower components are the more polar ones. This greater polarity could be caused by more sugars, other sugars or other groups on the aglycon with more oxygen atoms, for example an alcohol or acid group.

Saponin content in Maesa plants with different ages

Because *Maesa* species are shrubs and because seedlings or juvenile plants might be physiologically quite different from adult plants, we analyzed saponin content of greenhouse grown plants from approximately 1 month, 9 months and 2 years old. TLC results are shown in Figure 2-2.

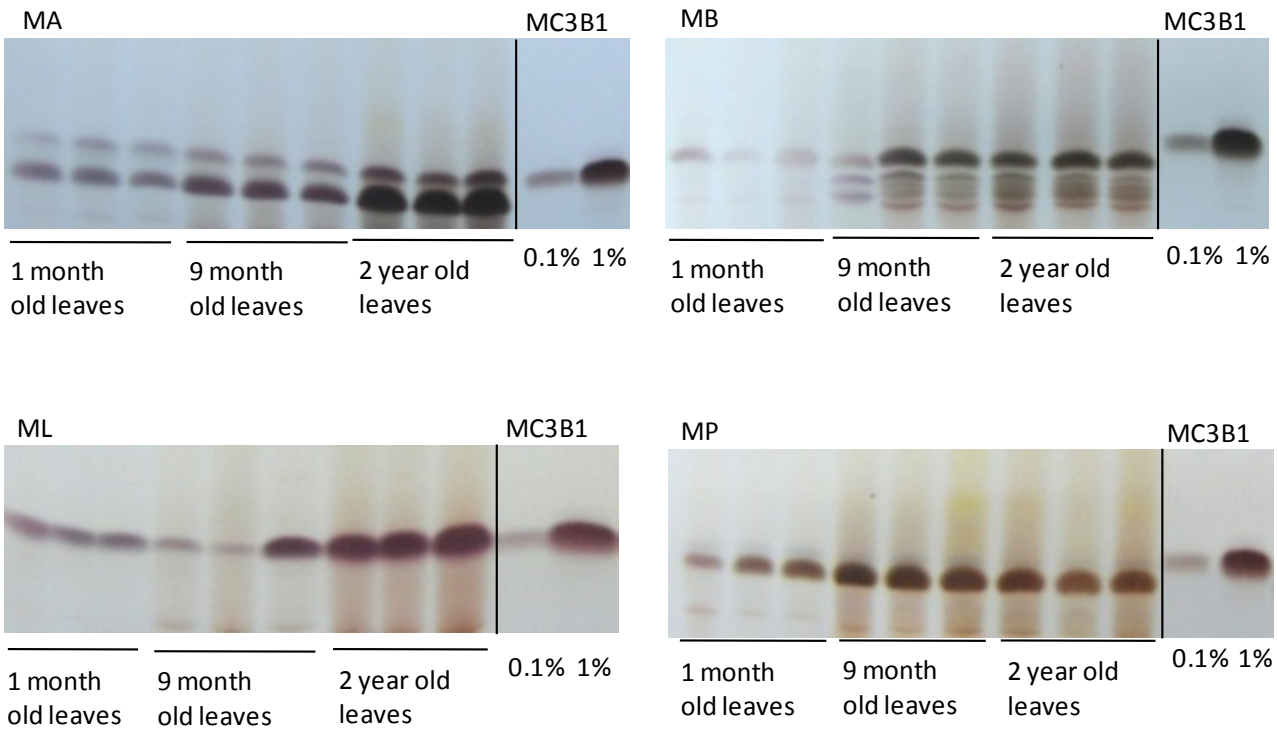


Fig 2-2 TLC analysis of saponin extracts from *M. argentea* (MA), *M. balansae* (MB), *M. lanceolata* (ML) and *M. perlarius* (MP) plants growing in the greenhouse for 1 month, 9 months and 2 years. A HPLC purified maesasaponin mix (MC3B1) was used as reference sample. Different repeats represent different plants.

For *M. argentea* the two characteristic bands, with R_f 0.17 and 0.14, were visible. For both spots, the intensity and surface increased as the age of the plants increased. For *M. balansae*, the spot with R_f 0.16 was more pronounced in leaves of 9 month and 2 year old plants, compared to 1 month old plants. Besides the band with R_f 0.16, smaller bands with higher retention factors were present. *M. lanceolata* had one band with R_f 0.17 and, although there was some variation between different plants of the same age, it was clear that leaves of 2 year old plants had a higher saponin content than leaves of the younger plants. For *M. perlarius* one large spot with R_f 0.16 was detected that was larger and more intense in 9 month and 2 year old plants. In addition, also a small blue band with a R_f of approximately 0.18 was visible. In general, we can conclude that saponin production in leaves of *Maesa* plants markedly increased with increasing age of the plants.

Saponin content of Maesa lanceolata leaves at different positions

From the previous experiment it was clear that there was a large variation in saponin content between plants of different ages. With the next experiment we wanted to test whether there was also variation in saponin content between leaves of the same plants at different positions (Figure 2-3). This experiment was performed with *M. lanceolata* plants of 2 years old.

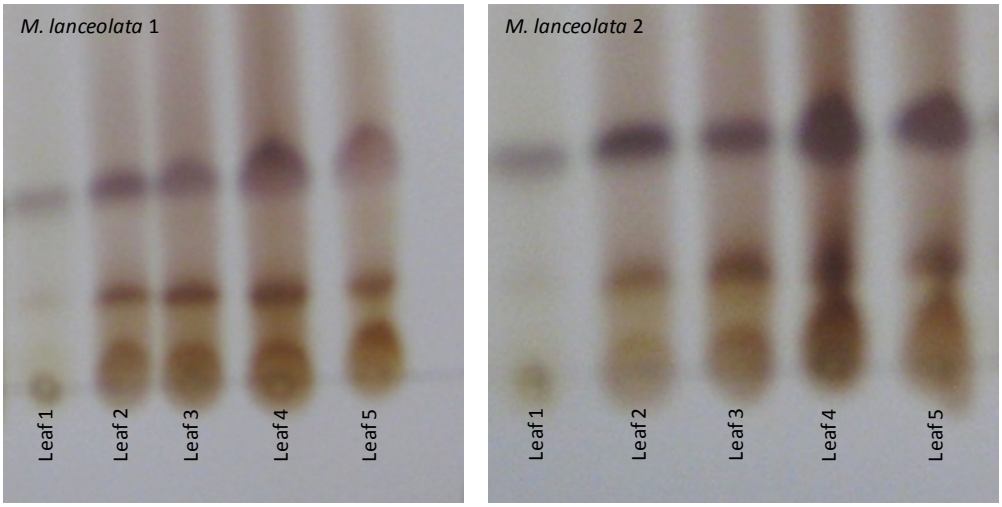


Fig 2-3 TLC of saponin extracts from leaves of 2 year old *M. lanceolata* plants. Leaves were collected from different positions on the plants. Every three leaves, one leaf was isolated, starting from the apex (leaf 1). Different repeats represent different plants.

One pink/purple saponin band with R_f 0.17 was detected in all *M. lanceolata* leaves. The TLC revealed that there was a correlation between the intensity of the spot and the distance of the leaf from the apex; the further the leaves were away from the apex, the higher the saponin content. So we can conclude that for *M. lanceolata* older and fully developed leaves had a higher saponin content than younger leaves near the apex.

Saponin content in leaves of Maesa plants after spraying with methyl jasmonate

To investigate whether *Maesa* saponins in greenhouse plants were inducible, we sprayed the plants with a 100µM MeJA solution. MeJA was dissolved in ethanol and therefore control plants were sprayed with ethanol. The leaves were harvested 48 hours after elicitor treatment. Samples were analyzed using TLC (Fig 2-4).

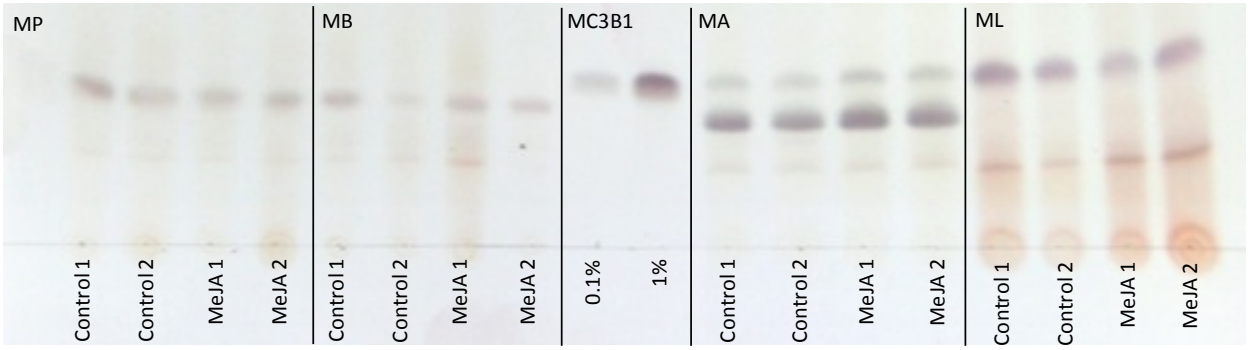


Fig 2-4 TLC analysis of leaves from greenhouse grown plants treated with 100µM MeJA. Controls were sprayed with ethanol and MC3B1 was used as a reference sample.

For all *Maesa* species, the same characteristic saponin bands were detected as in previous experiments. For none of these plants, though, we observed a change in saponin production after spraying with 100µM MeJA.

Injecting in vivo plants with methyl jasmonate (MeJA) and saponin precursors

Because spraying of *Maesa* plants with MeJA did not prove to have an effect on saponin production, we tried to inject the leaves with 100µM MeJA. In addition, we injected leaves with the saponin precursors squalene and farnesol, both in a concentration of 100µM. This experiment was performed only for *M. lanceolata* and we analyzed saponin content with TLC.

First, we tested if the injection itself could already have an effect on saponin production in the leaves. We injected with water and 0.4% ethanol, which is the solvent for both squalene precursors and MeJA, and compared the saponin content with non-treated leaves. We tested 3 different time points; 0, 24 and 96 hours (Fig 2-5).

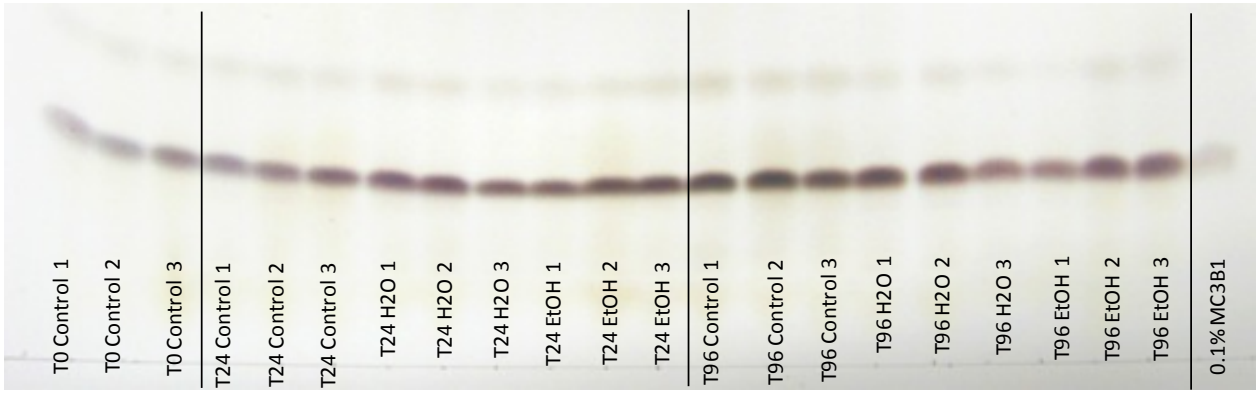


Fig 2-5 TLC analysis of *M. lanceolata* leaves injected with water and ethanol. Three different time points were tested 0 hours (T0), 24 hours (T24) and 96 hours (T96). Controls were non-treated leaves of the same plants. A HPLC purified maesasaponin mix (MC3B1) was used as reference sample. Different repeats represent different plants.

Injection of leaves with water or a low concentration of ethanol could already be a stress for the plant and could consequently result in changed saponin production. Though, none of the treatments seemed to have a clear effect on saponin production. Therefore, this injection method and solvent is appropriate to investigate the effect of injected MeJA and saponin precursors. First we tested the effect of 100µM MeJA and samples were harvested 48 hours after injection (Fig 2-6).

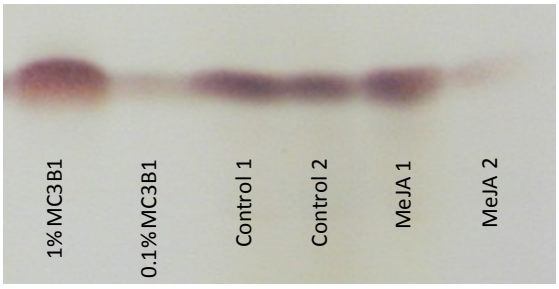


Fig 2-6 TLC analysis of *M. lanceolata* leaves injected with 100µM MeJA. Samples were harvested after 48 hours. Controls were non-treated leaves of the same plants. A HPLC purified maesasaponin mixture (MC3B1) was used as reference sample. Different repeats represent different plants.

TLC analysis of saponin extracts of *M. lanceolata* leaves injected with 100µM MeJA showed that there is no increase in saponin production. In one of the tested plants, treatment with MeJA led to an apparent decrease in saponin production. In a subsequent experiment it was tested if we could increase the saponin production through injection of saponin precursors, squalene and farnesol, in the leaves (Fig 2-7).

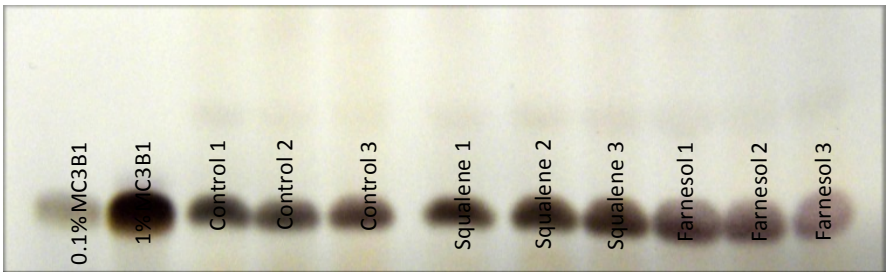


Fig 2-7 TLC analysis of *M. lanceolata* leaves injected with 100µM squalene or 100µM farnesol. Samples were harvested after 48 hours. Controls were non-treated leaves of the same plants. A HPLC purified maesasaponin mixture (MC3B1) was used as reference sample. Different repeats represent different plants.

TLC of *M. lanceolata* leaves injected with squalene and farnesol revealed a similar spot for all samples. This spot was darker for leaves treated with squalene. When comparing to the MC3B1 controls, this increase in saponin content is probably around 2-3 times. As a control, also pure squalene and farnesol solutions were tested on TLC. These did not give a colour reaction on the plate (results not shown).

2.4 Discussion

Using a miniaturized saponin extraction protocol and thin layer chromatography (TLC), we were able to investigate the saponin production in *Maesa* plants in greater detail. TLC plates were developed using an anisaldehyde reagent that coloured the saponins violet-blue. Probably, after spraying the reagent, a dehydration reaction occurs, forming unsaturated methylene groups which give colored condensation products with the aldehydes (Hostettmann and Marston 1995). Anisaldehyde reagent is, for instance, used for detection of triterpene saponins in *Quercus robur* and *Q. petraea* (Arramon et al. 2002), *Ilex kudingcha* (Tang et al. 2005), *Chenopodium quinoa* (Madl et al. 2006), *Aesculus hippocastanum* and *Vitis vinifera* (Apers et al. 2006).

TLC analyses of *Maesa* greenhouse grown plants revealed that saponins were present in leaves, stems and roots of *M. argentea, M. balansae, M. lanceolata* and *M. perlarius*. Though, there were differences in type and concentration of saponins between different organs. This was already shown for *M. lanceolata* by Foubert and coworkers; leaves of greenhouse grown plants contained 4.9% saponins, while roots of the same plants had 1.5% of saponins. In addition maesasaponin IV_2 was only detected in leaves, while maesasaponins IV_1 and V_1 were only detected in roots (Theunis et al. 2007). Also for *Panax ginseng*, differences in saponin content between different organs were observed. Ginsenoside levels in *Panax ginseng* were lowest in the leafstalks and stem (0.77%), intermediate in the main root (1.3%) and lateral roots (3.5%) and highest in the leaves (5.2%) and root hairs (6.1%) (Koizumi et al. 1982). Another

example is saponin production in oats (*Avena* spp.) that synthesize two different families of saponins. The distribution of these two classes of saponins is mutually exclusive; avenacosides (steroidal saponins) are produced in the leaves and avenacins (triterpenoid saponins) in the roots (Osbourn 2003).

Next to differences in saponin content in different organs, we also observed differences in saponin concentration depending on the developmental stage; older plants produced more saponins compared to younger plants and more mature leaves produced more saponins compared to smaller and younger leaves. Foubert et al. (2007) found, however, that leaves of plants growing in the greenhouse contained much more saponins than the leaves of an in nature grown and older tree, respectively 4.9% and 1.2%. They suggest that the higher saponin content in greenhouse plants could be due to an infection of the plants with woolly aphids. An increase in saponin content of *Medicago sativa* leaves was also reported after infection with *Spodoptera litteralis* (Agrell et al. 2003). Some of our plants were also infected with woolly aphids, however no difference in saponin content between infected and non-infected plants was observed (results not shown).

Methyl jasmonate (MeJA) is a plant hormone that is a widely used elicitor for induction of saponin production in plants (Creelman and Mullet 1997). For *Maesa* species, neither spraying nor injection of 100µM MeJA resulted in a drastic change in saponin production. This is in contrast with the numerous reports that describe higher saponin production upon treatment with MeJA, for example in *Panax ginseng* (Palazon et al. 2003)*, Medicago truncatula* (Suzuki et al. 2002)*, Centella asiatica* (Kim et al. 2004) and *Glycyrrhiza glabra* (Shabani et al. 2009). Besides elicitors, precursors are sometimes used to increase the secondary metabolite concentration in plants. Treatment with saponin precursors was for example successfully applied in *Panax ginseng* (Furuya et al. 1983; Linsefors et al. 1989; Sivakumar et al. 2006). In contrast, *Maesa lanceolata* plants treated with the precursors squalene and farnesol did not show large alterations in saponin production on TLC.

Taken together, these results point towards the production of saponins in *Maesa* species as phytoanticipins (constitutive phytoprotectants). Based on the following findings: (1) treatment of plants with a universal saponin elicitor, MeJA, and saponin precursors did not lead to a drastic increase in saponin production, (2) saponins were found in roots, shoots and stems of all species in relatively high concentrations and (3) there was a clear correlation between leaf developmental stage and saponin accumulation, we assume that maesasaponin production is mainly constitutive. Additional elicitation studies with *in vitro* plants further corroborate these conclusions (Chapter 4).

2.5 Materials and methods

2.5.1 Plant material

Maesa lanceolata seeds were collected in Moshi, Tanzania by Frank Mbago (Department of Botany, University of Dar-Es-Salaam). *Maesa balansae* and *Maesa perlarius* seeds were collected in the Trang Dinh district, Vietnam by Nguyen Tap (National Institute of Medicinal Materials Lang Son Province). Finally, *Maesa argentea* seeds (no. 61-2068) were provided by the National Botanical garden (Meise, Belgium).

The seeds were rinsed in 70% (v/v) ethanol (Chem-Lab NV) for 30 seconds and subsequently surface sterilized with a 70% (v/v) solution of a commercial disinfection product (Haz-tabs; Guest Medical, Kent, UK). After three washes with distilled water, the seeds were placed on Murashige-Skoog (MS) basal medium (Murashige and Skoog 1962) supplemented with 0.8% (w/v) agar (Lab M plant tissue culture agar MC29, Amersham) and 3% (w/v) sucrose (with pH 5.8). Seeds were germinated in a 16/8 h light/dark photoperiod at 26°C.

Two weeks after germination, plantlets were transferred to larger tissue culture containers (Meli-jars, (De Proft et al. 1985)) with MS medium supplemented with vitamins 0.7% (w/v) agar and 3% (w/v) sucrose (pH 5.8). The plants were transferred to fresh medium every month.

For acclimatization to greenhouse conditions, rooted plantlets were gently and thoroughly washed with water and subsequently transferred to 9 x 9 cm^2 small plastic pots containing a mixture of sand and peat soil (1:1) (groep AVEVE). The first 2-3 weeks, the plants were hardened in 100% humidity conditions at 25°C. Afterwards, they were transferred to normal greenhouse conditions at 25°C.

2.5.2 Crude saponin extraction

For miniaturized crude saponin extraction, 50mg fresh weight of plant material was ground with liquid nitrogen. 250µl of 50% (v/v) methanol (Chem-Lab NV) was added and samples were sonicated for 1 hour in a Bransonic 12 sonicator (117V, 60W). Subsequently, extracts were centrifuged at 2000rpm for 10 minutes (Heraeus® Biofuge® Primo R centrifuge) and the supernatant was transferred to a fresh Eppendorf tube. The pellet was resuspended with 250µl 50% (v/v) methanol and sonicated for another hour. The samples were again centrifuged for 10 minutes at 2000rpm and the supernatant was combined with the supernatant from the first extraction. Samples were dried for 3 – 4 hours using a vacuum concentrator (Heto VR-I, High Technology of Scandinavia) attached to a Savant RT4104 refrigerated condensation trap. When the samples were dried completely, the pellet was resuspended with 50µl of 80% (v/v) methanol. This extract was further used for TLC analysis.

2.5.3 Thin layer chromatography (TLC)

TLC analysis was performed with normal phase silica gel 60 plates with fluorescence indicator (F_{254}) (Merck KGaA, Germany). As mobile phase, the upper layer of an n-butanol/acetic acid/H_2O (40/10/50) mixture was used. One hour before starting the TLC run, the mobile phase was brought into the TLC tank and a filter paper was placed at the back of the tank. 10µl of the samples was spotted at 2cm from the sides and bottom of the plate. Afterwards, the plate was placed in the tank for 5 hours. The composition of the anisaldehyde reagent used for detection of saponins on the TLC was: 0.5ml/l p-anisaldehyde (99% purity, Acros Organics), 100ml/l 100% (v/v) acetic acid (99% purity, Carl Roth GmbH), 850ml/l 100% (v/v) methanol (99% purity, Chem-Lab NV) and 50ml/l 100% (v/v) sulphuric acid (99-100% purity, UCB). The reagent was sprayed onto the TLC plate using an EcoSpray (Carl Roth GmbH). Saponin spots should be visible 10 minutes after heating the plate to 100°C on a hot plate (Stuart® SD160 digital hotplate). A HPLC purified maesasaponin mixture (MC3B1) was used as a reference sample on TLC.

2.5.4 Saponin studies in *Maesa* greenhouse plants

Saponin content in Maesa leaves, stems and roots

For crude saponin extraction, three different 1-month-old greenhouse grown plants were used for each *Maesa* species. The plants were removed from the soil and were rinsed gently with water. Subsequently all leaves, stems and roots were collected from each plant and used for further saponin analysis.

Saponin content in Maesa plants with different ages

In this experiment, we used from each *Maesa* species three 1 month old, three 9 months old and three 2 years old greenhouse grown plants. From these plants 2nd and 3rd leaves (starting from the apex) were combined and used for saponin extraction.

Saponin content of Maesa lanceolata leaves at different positions

Leaves from two different 2 years old greenhouse grown *Maesa lanceolata* plants were isolated from 5 different positions. Every three leaves, we took one leaf (starting from the apex). This leaf material was used for saponin extraction.

Saponin content in leaves after spraying with methyl jasmonate

Second and third leaves of 1 month old greenhouse grown *Maesa* plants were sprayed with 100% (v/v) ethanol (solvent for MeJA) or 100µM MeJA (95% purity, Sigma Aldrich) until run-off. Sprayed leaves were harvested (2nd and 3rd leaves of the same plant were combined) after 48 hours and were immediately ground with liquid nitrogen en further processed for saponin extraction. For each species, two different plants were used.

Injecting leaves with methyl jasmonate and saponin precursors

First, a control experiment was performed to investigate the effect of the injection itself. *M. lanceolata* 2nd and 3rd leaves of 1 month old greenhouse grown plants were injected with water or 0.4% (v/v) ethanol (solvent for MeJA, squalene and farnesol) using a 10ml syringe (BD bioscience). The injected area was indicated and leaves were harvested at three time points; 0, 48 and 96 hours. The injected parts of the leaves were then ground with liquid nitrogen and saponins were extracted (2nd and 3rd leaves from the same plant were combined). This experiment was performed in three repeats, i.e. with three different plants.

For the second experiment, the same method was used to inject 2nd and 3rd leaves of 1 month old *M. lanceolata* plants with 100µM MeJA (95% purity, Sigma Aldrich), 100µM squalene (98% purity, Sigma Aldrich) and 100µM farnesol (95% purity, mixture of isomers, Sigma Aldrich. Non-treated samples were used as controls. Samples were harvested 48 hours after treatment. Injected parts of the leaves were used for saponin extraction. The experiment with MeJA was done with two repeats, the experiment with the precursors was done in triplicate.

CHAPTER 3

IN VITRO CONSERVATION AND MICROPROPAGATION OF MAESA SPECIES

Parts of this chapter have been published as:

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3.1 Abstract

To circumvent problems with conventional propagation of *Maesa* spp. and to obtain plant material suitable for future experiments, different types of *in vitro* cultures were established. Callus was induced for all four species on medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) alone or in combination with a cytokinin or other auxin. For *M. lanceolata* also cell cultures were initiated and growth characteristics of calli and cell suspensions were analyzed. In addition, successful micropropagation systems were developed for four *Maesa* species. Multiple shoots were induced through both axillary bud formation and adventitious shoot regeneration from leaf explants. Also callus material could be regenerated to shoots. The explants were cultured on medium supplemented with 6-benzyladenine (BA), thidiazuron (TDZ) and/or α-naphthalene acetic acid (NAA). The success of regeneration varied for different species and depended on the type and concentration of plant growth regulators. Regenerated shoots spontaneously developed roots within 6 weeks on MS hormone-free medium. The rooted shoots were transferred to the greenhouse with a very high success rate. These protocols enable us to induce multiple clonal shoots that easily root and acclimatize to greenhouse conditions from relatively small amounts of starting material. Furthermore, flow cytometry analysis indicated that there were no changes in ploidy level of axillary and adventitious shoots as compared with wild type adult plants. Plants regenerated from callus, however, did show polyploidization. Thin layer chromatography (TLC) analysis revealed that common and distinguishing spot of saponins were similarly observed in regenerated shoots compared to the control plants. Therefore, the developed protocols also provide an effective means for the *in vitro* conservation of *Maesa* spp. that produce pharmaceutically interesting saponins.

3.2 Introduction

In vitro tissue culture techniques have become a feasible alternative to improve the efficiency of propagation of medicinal plants as well as to facilitate *in vitro* experiments such as genetic transformation, protoplast fusion and investigation of secondary metabolites biosynthesis gene expression. *In vitro* culture technology has already been implemented for propagation of many medicinal plants (reviewed in Debnath et al. 2006 and Rout et al. 2000). Medicinal plants are often grown under *in vitro* conditions to conserve the germplasm. For example *Cecropia* spp. (Nicasio-Torres et al. 2009), *Ceropegia intermedia* (Karuppusamy et al. 2009), *Bacopa monnieri* (Singh et al. 2009), *Asparagus racemosus* (Bopana and Saxena 2008) and *Searsia dentata* (Prakash and Van Staden 2008) are propagated and stored *in vitro*. In some cases, tissue and cell culture are developed for the production of interesting secondary metabolites. For example, *in vitro* root cultures of *Catharanthus roseus* are used for the production of two anti-cancer alkaloids, vinblastine and vincristine (Ataei-Azimi et al. 2008). Cell

cultures of *Taxus* spp. are used to produce large amounts of alkaloid taxol. Taxol is a chemotherapeutic agent, approved in the treatment of a variety of cancers. Taxol is currently supplied through both a semi-synthetic process and plant cell culture (Vongpaseuth and Roberts 2007).

In this chapter, different types of *in vitro* cultures are evaluated for the micropropagation of the medicinal plants *Maesa argentea, M. balansae, M. lanceolata* and *M. perlarius*. Flow cytometry was used to measure DNA content in regenerated plants and to check potential occurrence of somaclonal ploidy variation. In addition, saponin content of regenerated plants was investigated using thin layer chromatography (TLC).

3.3 Results

3.3.1 In vitro initiation of *Maesa* species

Aseptic *Maesa* shoot cultures were initiated from seedlings. *Maesa* seeds germinated in 6 - 8 weeks. The percentage of seeds that germinated was around 30% for *M.* argentea, 50 – 70% for *M. balansae* and *M. lanceolata* and 90% for *M. perlarius*. After 4 weeks, seedlings were brought into larger tissue culture containers and were subcultured every two months (Fig 3-1).

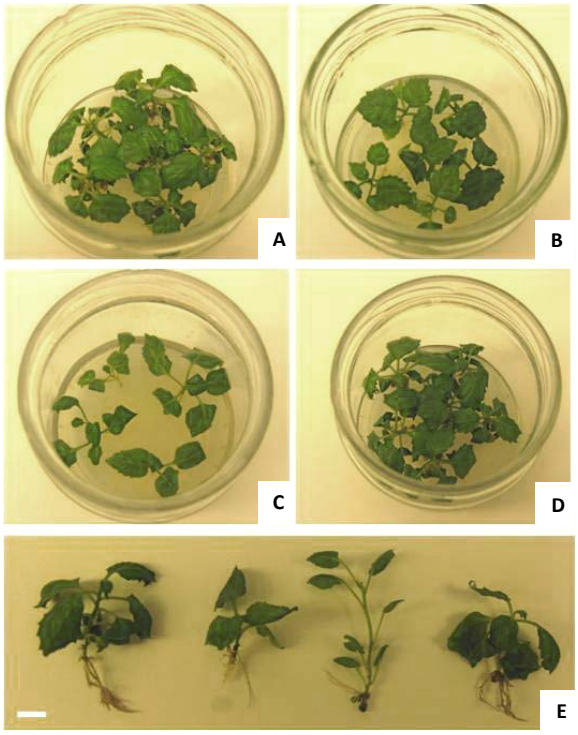


Fig 3-1 Overview of *Maesa* plantlets *in vitro*: *M. argentea* (**a**)*, M. balansae* (**b**), *M. lanceolata* (**c**) and *M. perlarius* (**d**). Picture **e** from left to right: *M. argentea, M. balansae, M. lanceolata, M. perlarius.* Bar = 1 cm.

3.3.2 Multiplication through axillary shoot formation

Shoot induction

A micropropagation protocol using axillary shoot formation was described for *Maesa ramentacea* (Kanchanapoom and Boonvanno 2000). We tested a similar protocol for *in vitro* culturing of four *Maesa* species of our interest. The hormone concentrations supplemented to the medium were optimized for multiplication of *Maesa argentea, M. balansae, M. lanceolata* and *M. perlarius* and are summarized in Table 3-1.

Table 3-1 Influence of the plant growth regulators (PGRs) BA and NAA on axillary bud multiplication in different *Maesa* species

PGRs (µM)		*M. argentea*	*M. balansae*	*M. lanceolata*	*M. perlarius*
BA	NAA	# buds/explant (± SE)	# buds/explant (±SE)	# buds/explant (± SE)	# buds/explant (± SE)
-	-	1.0 ± 0.0^{d}	1.4 ± 0.3^{bc}	1.3 ± 0.2^{c}	2.2 ± 0.8^{abc}
4.4	-	3.0 ± 0.4^{bc}	2.2 ± 0.2^{ab}	1.7 ± 0.3^{c}	2.7 ± 0.5^{ab}
8.8	-	4.7 ± 0.3^{b}	2.7 ± 0.2^{a}	4.7 ± 0.7^{ab}	2.8 ± 1.9^{bc}
13.2	-	**6.7 ± 1.1^{a}**	1.8 ± 0.5^{abc}	6.3 ± 1.2^{a}	3.2 ± 0.4^{a}
4.4	10.7	0.7 ± 0.2^{d}	1.3 ± 0.2^{bc}	0.8 ± 0.3^{c}	0.5 ± 0.2^{c}
13.2	13.5	0.0 ± 0.0^{d}	1.0 ± 0.0^{c}	1.2 ± 0.2^{c}	0.3 ± 0.2^{c}
22.2	5	1.5 ± 0.2^{cd}	2.0 ± 0.6^{abc}	2.8 ± 0.3^{bc}	3.8 ± 0.9^{a}

Experiment was performed in triplicate with at least four plants per replicate. Different letters indicate significant differences ($p<0.05$) within one species, according to Tukey test.

The effect of different hormones on the induction of shoots depended on the species. *M. argentea* and *M. lanceolata* produced the highest number of shoots, 6.7 and 6.3 respectively, with BA alone at a concentration of 13.2 µM. For *M. lanceolata* there was, however, no statistical significant difference between treatment with 8.8 µM or 13.2 µM BA. *M. balansae* formed an average of 2.7 axillary buds per explant after treatment with 8.8 µM BA. Other hormone treatments did not show a significant difference with the control. For *M. perlarius* none of the hormone treatments gave a significant difference compared to the hormone free control medium. Notably, the multiplication rate of the control plants of *M. perlarius* was higher than that of the controls of the other species.

Rooting and acclimatization

The axillary buds elongated and developed roots in basal MS medium without phytohormones with an efficiency of 100% (Table 3-2). For acclimatization, rooted shoots were hardened also with a 100%

efficiency rate (Table 3-2). After transfer to the greenhouse, the plantlets continued to grow and develop into normal and vigorously growing plants.

Table 3-2 Rooting and acclimatization of shoots initiated through axillary bud multiplication for all four *Maesa* species.

Sample		Rooting		Acclimatization	
Species	Explant type	# samples	% rooting	# samples	% acclimatization
M. argentea	Axillary bud	30	100	15	100
M. balansae	Axillary bud	30	100	15	100
M. lanceolata	Axillary bud	30	100	15	100
M. perlarius	Axillary bud	30	100	15	100

Ploidy analysis

Inducing adventitious shoots through *in vitro* regeneration is sometimes accompanied by genetic instability also known as somaclonal variation (Larkin and Scowcroft 1981). To further investigate the impact of tissue culturing on the plant genome stability, regenerated shoots were analyzed. The variations that are commonly noticed are the ploidy level, chromosome structure, mitotic abnormalities and other cytological disorders (Radić et al. 2005). The ploidy level of *Maesa* regenerated shoots was determined through flow cytometric analysis of leaves and compared to the ploidy level of greenhouse grown plants. The flow cytometry analysis showed similar peaks in all samples (Fig 3-2). No significant changes in DNA content were observed for any of the *in vitro* propagated *Maesa* species compared to the greenhouse grown plants. We therefore conclude that the ploidy level was maintained at the diploid status during *in vitro* culturing and the regeneration process.

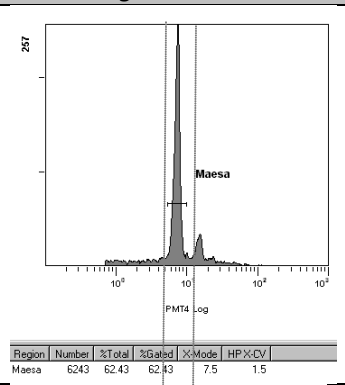
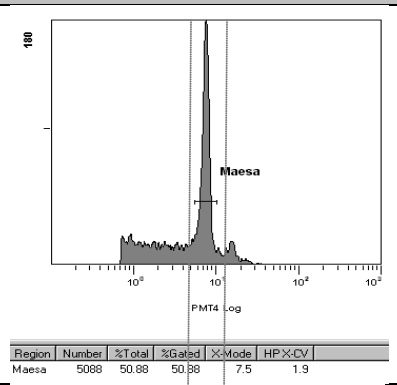
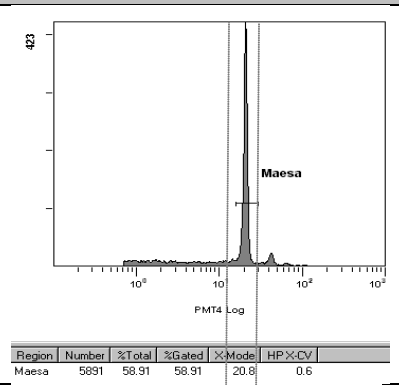
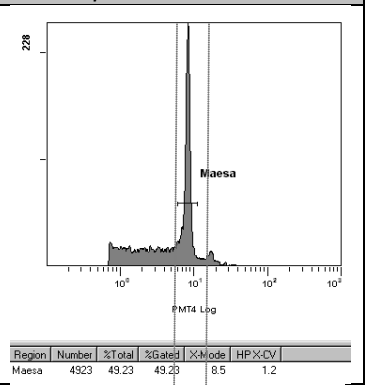
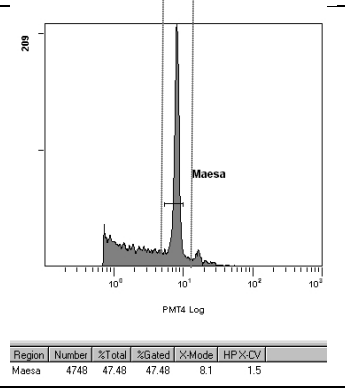
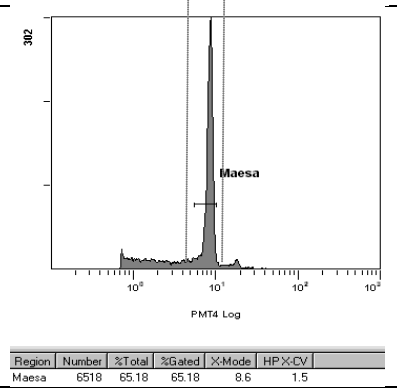
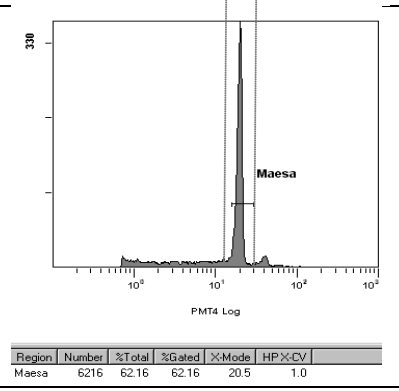
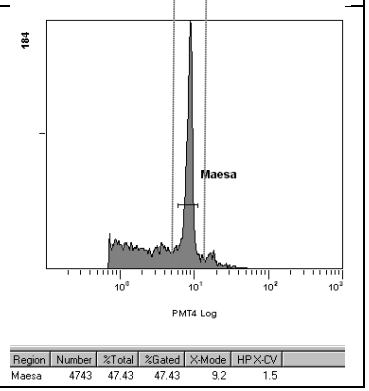
	Maesa argentea	*Maesa balansae*	*Maesa lanceolata*	*Maesa perlarius*
Greenhouse plants				
Axillary buds				

Fig 3-2 Histograms of relative fluorescence intensity (log-transformed, PMT4 log) of isolated nuclei from four *Maesa* species regenerated through axillary shoot multiplication compared to control greenhouse grown plants

3.3.3 Callus induction and growth

Callus induction

For callus induction, *in vitro* leaves of all four species were placed on solid MS medium supplemented with auxin and cytokinin. Tested hormone combinations for callus induction on leaves of *Maesa argentea, M. balansae, M. lanceolata* and *M. perlarius* are represented in Table 3-3.

Table 3-3 Effect of plant growth regulators (PGRs) on the induction of callus on the leaf material of different *Maesa* species. Callus formation was evaluated after 6 weeks.

PGRs (μM)				*M. argentea*		*M. balansae*		*M. lanceolata*		*M. perlarius*	
NAA	BA	2,4-D	Kin	Callusing	Morph.	Callusing	Morph.	Callusing	Morph.	Callusing	Morph.
-	-	-	-	no		no		no		no	
2.70	-	-	-	no		no		no		no	
-	4.40	-	-	no		no		no		no	
-	-	5.00	-	no		no		**yes**	**WG**	**yes**	**BS**
-	-	-	0.46	no		no		no		no	
2.70	4.40	-	-	no		no		no		no	
2.70	-	5.00	-	no		**yes**	**YG**	**yes**	**WG**	**yes**	**WS**
2.70	-	-	0.46	no		no		no		no	
-	4.40	5.00	-	**yes**	**BG**	**yes**	**YG**	**yes**	**WG**	**yes**	**BG**
-	4.40	-	0.46	no		no		no		no	
-	-	5.00	0.46	**yes**	**BG**	**yes**	**BS**	**yes**	**YG**	**yes**	**WS**

Experiments were performed in triplicate and callusing was always observed in each replicate. **B** = brown, **Y** = yellow, **W** = white, **G** = granular, **S** = smooth

Six weeks after induction, callus was observed for all four species. Results suggest that the auxin 2,4-D is necessary for callus induction. For *M. lanceolata* and *M. perlarius*, callus was observed when 2,4-D was applied alone or in combination with NAA, BA or kinetin. On *M. balansae* leaves callus was induced when 2,4-D was applied in combination with NAA, kinetin or BA and for *M. argentea* only callus was produced when 2,4-D was applied together with a cytokinin. Different types of calli were observed and differences were mostly species specific. *M. argentea* calli showed a brown color while callus of *M. lanceolata* had a white – yellowish color and was granular. In contrast, callus of *M. perlarius* was often very smooth and had a cream white to brown color. For *M. balansae* there was more variation in callus morphology, as well smooth as granular callus was found.

Callus maintenance and growth

Callus was subcultured every month and put on fresh medium with the same composition as the medium that was used for the induction. A growth curve for all types of callus was established by weighing the calli every week (Fig 3-3).

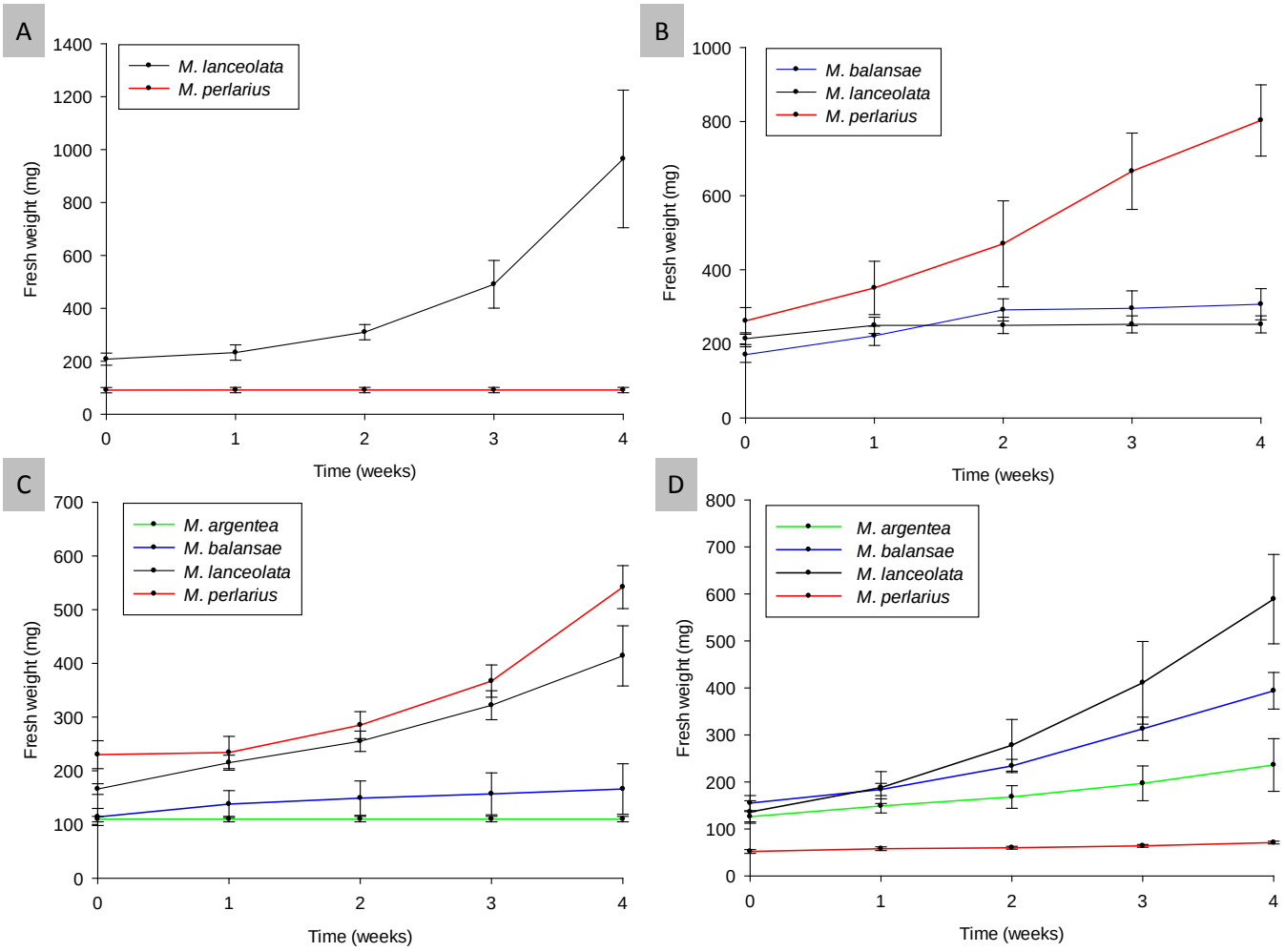


Fig 3-3 Growth curve for callus of four *Maesa* species on culture medium with four different hormone combinations; 5µM 2,4-D (**a**), 5µM 2,4-D and 2,7µM NAA (**b**), 5µM 2,4-D and 0.46µM Kinetin (**c**), 5µM 2,4-D and 4.4µM BA (**d**). The weight of the calli was followed over four weeks. The values are mean values of three independent calli; the error bars represent the standard error between repeats.

M. argentea and *M. balansae* callus seemed to grow well only on medium with 2,4-D and BA (Fig 3-3, green lines for *M. argentea* and blue lines for *M. balansae*). *M. lanceolata* callus showed a good growth rate on both medium with 2,4-D alone and medium with 2,4-D and cytokinins (Fig 3-3, black lines).

Finally, for *M. perlarius* callus the culture medium with 2,4-D in combination with NAA and kinetin was best (Fig 3-3, red lines). These good growing callus cultures were used in further experiments.

3.3.4 Establishment of cell suspensions

For *M. lanceolata,* cell cultures were initiated using friable callus growing on medium with 2,4-D and kinetin. The growth characteristics of the cell cultures could be determined by calculating the packed cell volume (PCV) every 2 - 3 days (Fig 3-4).

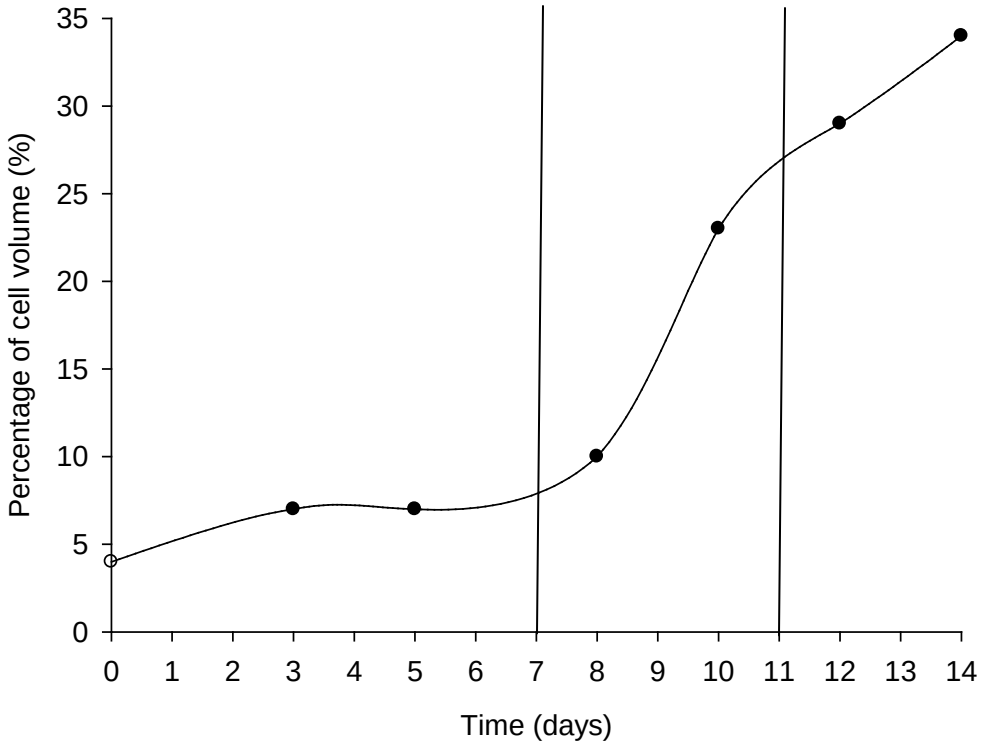


Fig 3-4 Growth curve of *M. lanceolata* cell suspensions. Three different growth phases could be distinguished. The cell suspensions were subcultured at day 14. The graph represents the accumulated cell volume of a single measurement of a typical *M. lanceolata* cell suspension culture.

Classically, three explicit growth phases can be distinguished during the culture of cell suspensions. The initial growth phase is called the lag phase and during this phase, the percentage of cell volume approximately stays the same. For *M. lanceolata* the lag phase lasted until the 7th day after subculturing. Subsequently the cells start to grow and divide continuously and the cell volume increases exponentially. This phase is called the exponential phase and for *M. lanceolata* this took place between day 7 and day

11 post subculturing. Normally the exponential phase is followed by a stationary phase in which the cell volume does not change anymore. In *M. lanceolata* cell suspensions the cell volume still increased from day 11 until day 14, however, the increase was less strong compared to the exponential phase. At day 14, the cell suspensions were subcultured. Other *M. lanceolata* cell cultures also showed at day 7-8 the onset of an exponential phase that lasted about 4-5 days. However, some of the suspensions did not exhibit a distinct exponential growth phase.

Besides these cell cultures that grew well, we also had many cultures that turned brown and arrested growth after a random period of subculturing. The reason for this phenomenon was not clear. The discontinuity of the growth behavior of the suspension cultures prompted us to abandon further investigations.

3.3.5 Callus regeneration

To test the regenerative capacity of *Maesa* callus, we examined if the callus material could be stimulated to develop into shoots. In addition, regeneration of shoots from callus is another micropropagation method that could be used for massive clonal propagation of *Maesa* species.

Shoot induction

For this regeneration experiment, 1 year old callus of all four *Maesa* species was placed on solid medium with 15 different BA and kinetin concentration and incubated in a growth room with 16/8h light/dark condition and 25°C. Six weeks after incubation, the calli had grown but did not yet show signs of shoot formation. In parallel with these tests, experiments were performed to induce adventitious shoots on *Maesa* leaf material and it was found that TDZ in combination with NAA gave better results than BA or kinetin (described in § 3.3.6). Therefore, calli on regeneration medium were split in two; half of the callus was placed on fresh medium with the same concentration of BA and/or kinetin and the other half of the callus was placed on medium with 4.5µM TDZ and 0.05µM NAA (Table 3-4).

Table 3-4 Shoot induction on callus of *M. argentea, M. balansae, M. lanceolata* and *M. perlarius.* Callus was first incubated on medium with BA and kinetin in different concentrations. After 6 weeks half of the calli were placed on medium with 4.5µM TDZ and 0.05µM NAA. First shoots were observed 17 weeks after the beginning of the experiment.

PGRs (µM)				*M. argentea*		*M. balansae*		*M. lanceolata*		*M. perlarius*	
BA	Kin	TDZ	NAA	Roots	# shoots/ callus ±SE	Roots	# shoots/ callus ±SE	Roots	# shoots/ callus ±SE	Roots	# shoots/ callus ±SE
-	-	-	-	Yes	**5.2 ±1.1**[a]	Yes	0.0[a]	No	0.0[a]	No	0.0[a]
0.44	-	-	-	Yes	0.0[b]	Yes	0.0[a]	No	0.0[a]	No	0.0[a]
2.22	-	-	-	Yes	0.0[b]	Yes	0.0[a]	No	0.0[a]	No	0.0[a]
4.44	-	-	-	Yes	0.0[b]	Yes	0.0[a]	No	0.0[a]	No	0.0[a]
-	0.46	-	-	Yes	0.0[b]	Yes	0.0[a]	No	0.0[a]	No	0.0[a]
-	2.32	-	-	Yes	0.0[b]	Yes	0.0[a]	Yes	0.0[a]	No	0.0[a]
-	4.64	-	-	Yes	0.0[b]	Yes	0.0[a]	No	0.0[a]	No	0.0[a]
0.44	0.46	-	-	Yes	0.0[b]	Yes	0.0[a]	Yes	0.0[a]	No	0.0[a]
0.44	2.32	-	-	Yes	0.0[b]	Yes	0.0[a]	No	0.0[a]	No	0.0[a]
0.44	4.64	-	-	Yes	0.0[b]	Yes	0.0[a]	No	0.0[a]	No	0.0[a]
2.22	0.46	-	-	Yes	0.0[b]	Yes	0.0[a]	Yes	0.0[a]	No	0.0[a]
2.22	2.32	-	-	Yes	0.0[b]	Yes	0.0[a]	No	0.0[a]	No	0.0[a]
2.22	4.64	-	-	Yes	0.0[b]	Yes	0.0[a]	No	0.0[a]	No	0.0[a]
4.44	0.46	-	-	Yes	0.0[b]	Yes	0.0[a]	Yes	0.0[a]	No	0.0[a]
4.44	2.32	-	-	Yes	0.0[b]	Yes	0.0[a]	No	0.0[a]	No	0.0[a]
4.44	4.46	-	-	Yes	0.0[b]	Yes	0.0[a]	Yes	0.0[a]	No	0.0[a]
-	-	4.5	0.05	Yes	1.6 ±0.4[b]	Yes	**0.2 ±0.1**[a]	No	0.0[a]	Yes	**0.4 ±0.2**[a]

The experiment was performed in triplicate with 9 repeats per replicate. Different letters indicate significant differences (P > 0.05) within one species; according to Tukey test.

17 weeks after the start of the experiment, none of the calli on medium with BA and/or kinetin showed induction of shoots. Some of the calli turned green suggesting that chlorophyll was synthesized. However, the calli on medium with TDZ and NAA appeared darker green than calli on medium with BA and kinetin. The chlorophyll content of the calli was determined and results are represented in Table 3-5. For *M. argentea* and *M. balansae* there was a significant increase in chlorophyll concentration of calli incubated on medium with TDZ compared to controls and calli on medium with BA and kinetin. For *M. lanceolata* and *M. perlarius* the increase in chlorophyll in calli on TDZ/NAA medium was not significantly different from calli grown on BA/kinetin medium. Though, there was a significant difference compared to the controls on hormone-free medium (Table 3-5).

Table 3-5 Total chlorophyll content of calli incubated on regeneration medium with different growth regulators.

PGRs	Total chlorophyll content in calli (mg/g fresh weight)			
	M. argentea	*M. balansae*	*M. lanceolata*	*M. perlarius*
None	0.06 ± 0.01[b]	0.02 ± 0.005[c]	0.03 ± 0.004[b]	0.02 ± 0.006[b]
BA and Kin	0.09 ±0.02[b]	0.08 ± 0.020[b]	0.09 ± 0.030[a]	0.09 ± 0.030[a]
TDZ and NAA	0.21 ±0.06[a]	0.14 ± 0.040[a]	0.11 ± 0.040[a]	0.14 ± 0.060[a]

Represented values are the mean of three independent chlorophyll measurements. Different letters indicate significant differences (P > 0.05) within one species; according to Tukey test.

Next to increased chlorophyll synthesis, some calli on medium with BA and kinetin also produced roots (Table 3-4, Fig 3-5). Roots were induced with all hormone concentrations for *M. argentea* and *M. balansae*. *M. lanceolata* calli only formed roots on some media and for *M. perlarius* no root growth was observed. Root growth was also observed on medium with TDZ and NAA, except for *M. lanceolata* calli.

As already mentioned, medium with BA or kinetin did not induce shoots. On medium with 4.5 µM TDZ and 0.05 µM NAA callus regeneration was observed for *M. argentea, M. balansae* and *M. perlarius* (Table 3-3, Fig 3-5). The formation of shoots was macroscopically visible after 17 weeks of incubation. *M. argentea* calli were most efficient in forming shoots with, on average, 1.6 shoots per callus. Even on hormone-free medium *M. argentea* callus produced a large number of shoots, namely 5.2 per callus. *M. balansae* and *M. perlarius* calli produced shoots on TDZ/NAA medium with a lower frequency than *M. argentea*, namely 0.2 and 0.4 shoots per callus, respectively. For *M. lanceolata* none of the treatments allowed the induction of shoot formation.

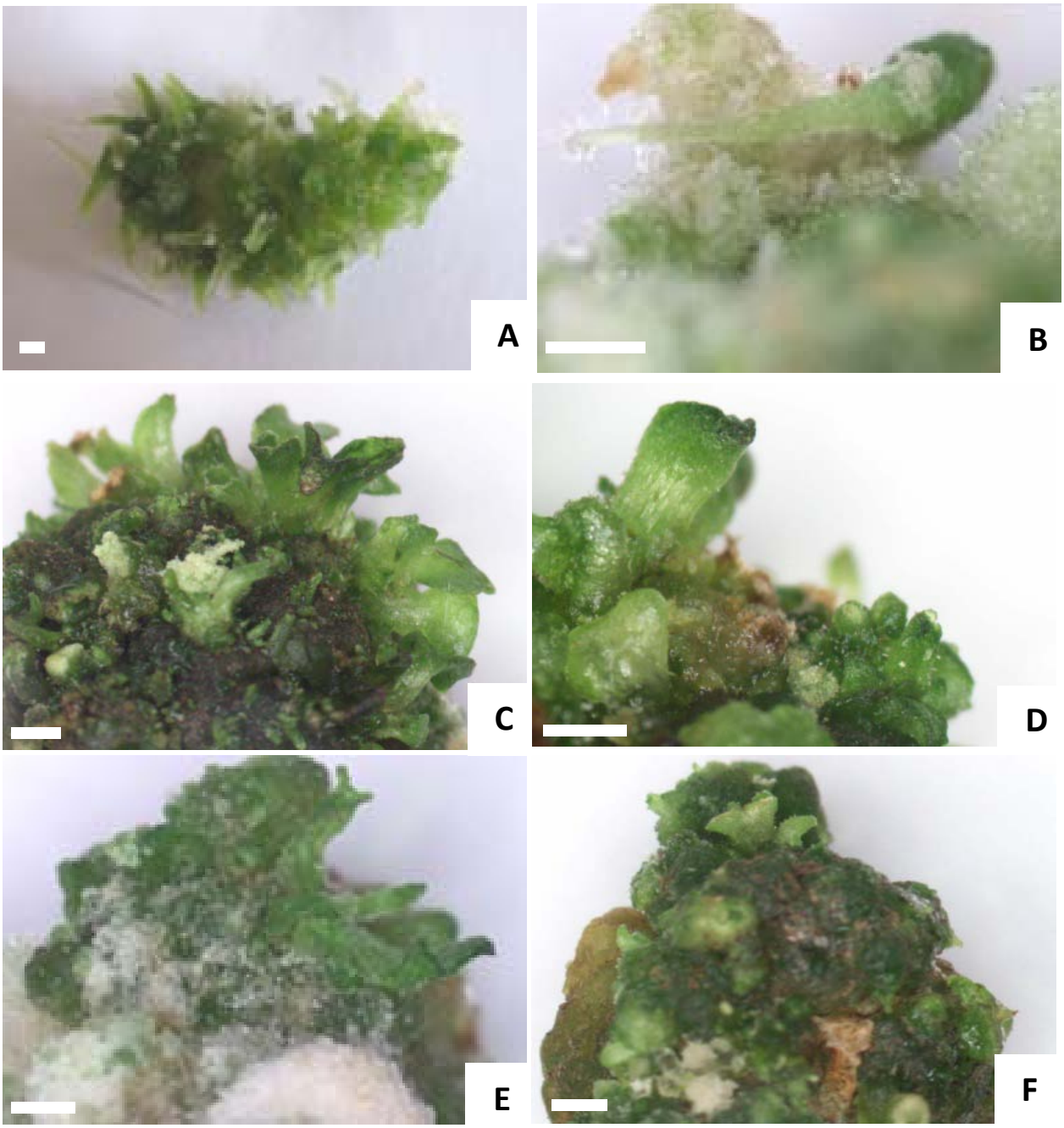


Fig 3-5 Roots induced on callus of *M. balansae* on medium with BA and kinetin (**a**) and on medium with TDZ and NAA (**b**). Shoots are induced on callus of *M. argentea*, as well on hormone-free medium (**c**) as on medium with TDZ and NAA (**d**). For *M. balansae* (**e**) and *M. perlarius* (**f**) shoots were induced on medium with TDZ and NAA. Bar = 1mm.

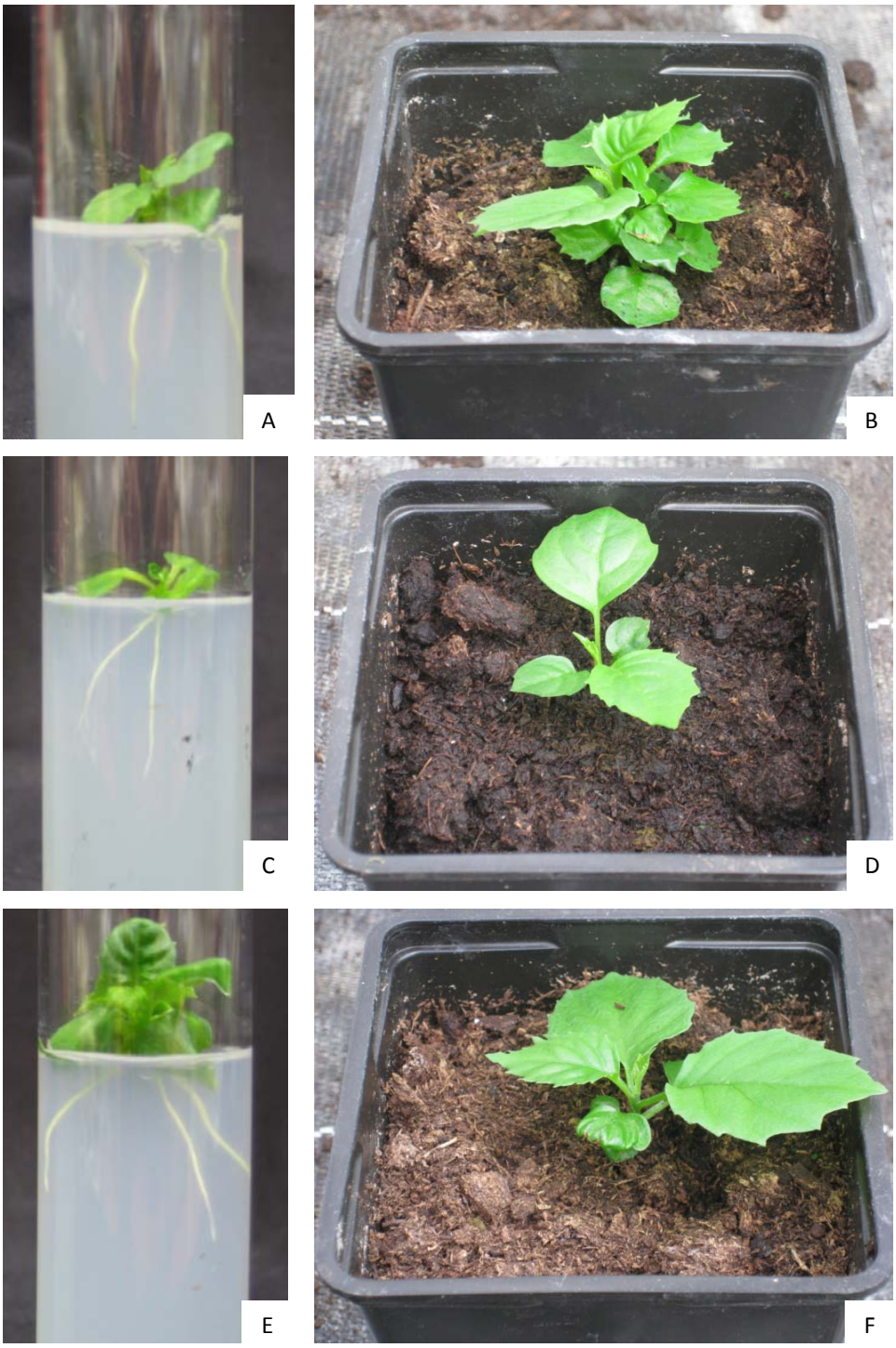
Rooting and acclimatization

Shoots were isolated from the calli and placed on basal MS medium without hormones for rooting. Roots spontaneously emerged after 8 weeks with an efficiency of 100 % for *Maesa argentea* and *M. perlarius* and 38% for *M. balansae* (Table 3-6, Fig 3-6). Plantlets with a good rooting system were transferred to the greenhouse. Hardening was successful for 88% of the *M. argentea* shoots, 67% of the *M. balansae* shoots and 50% of the *M. perlarius* shoots (Table 3-6, Fig 3-6).

Table 3-6 Rooting and acclimatization of shoots initiated through callus regeneration for all *M. argentea, M. balansae* and *M. perlarius*.

Sample		Rooting		Acclimatization	
Species	Explant type	# samples	% rooting	# samples	% acclimatization
M. argentea	Shoot from callus regeneration	65	100	8	88
M. balansae	Shoot from callus regeneration	22	100	6	67
M. perlarius	Shoot from callus regeneration	13	38	6	50

It was noticed that *M. balansae* regenerated shoots grew less well than *M. argentea* and *M. perlarius* shoots. Consequently, not enough plant material was available for genetic and biochemical studies on *M. balansae* shoots and further experiments focused on the two other species.



F**ig 3-6** Rooting of *in vitro* regenerated shoots of *M. argentea* (**a**), *M. balansae* (**c**) and *M. perlarius* (**e**). Pictures were taken 8 weeks after culture on MS basal medium without hormones. Shoots with good developed roots were transferred to the greenhouse. Pictures show shoots of *M. argentea* (**b**), *M. balansae* (**d**) and *M. perlarius* (**e**), 6 weeks after transfer to greenhouse condition.

Ploidy analysis

M. argentea and *M. perlarius* regenerated plants were first analyzed on a genetic level and flow cytometry was used to measure the ploidy levels of regenerated plants (Fig 3-7).

A clear shift was observed for the fluorescence peak of *M. argentea* and *M. perlarius* regenerants compared to control plants. Relative DNA content of controls and regenerants is presented in Table 3-7.

Table 3-7 X-values of *Arabidopsis thaliana,* controls and regenerants of *M. argentea* and *M. perlarius*. Relative DNA content was calculated for *M. argentea* and *M. perlarius* controls and regenerants based on the 2C nuclear DNA content of *A. thaliana* and X-values. Also the ratio in DNA content between controls and regenerants is represented.

Plant material		X-value	Absolute DNA content (pg)	Ratio control/regenerant
A. thaliana	2C	1.2	0.3	
M. argentea	Controls	5.9	1.5	2.1
	Regenerants	12.3	3.2	
M. perlarius	Controls	6.7	1.7	1.8
	Regenerants	12.5	3.1	

A. thaliana has an X-value of 1.2 and a 2C nuclear DNA content of 0.3 pg (Arumuganathan and Earle 1991). By means of these data, the absolute DNA content of *M. argentea* and *M. perlarius* plantlets could be calculated. DNA content of control *M. argentea* and *M. perlarius* plants was approximately 5 times higher than for *A. thaliana.* More importantly, the nuclear DNA content of regenerants is about 2 times that of the controls which is a clear proof of the polyploid status of the regenerated plantlets. Callus material of both species also showed an increase in DNA content (data not shown). We therefore conclude that the polyploidization of *M. argentea* and *M. perlarius* occurred during callus induction and/or culturing. Hence, regeneration of these calli resulted in polyploid plantlets.

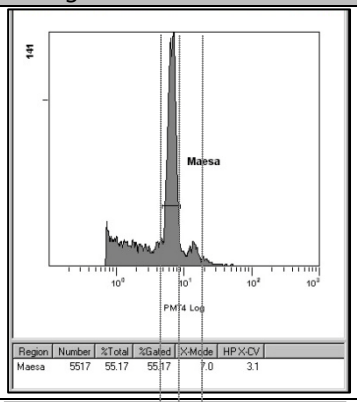
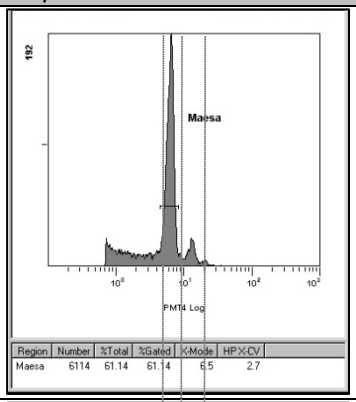
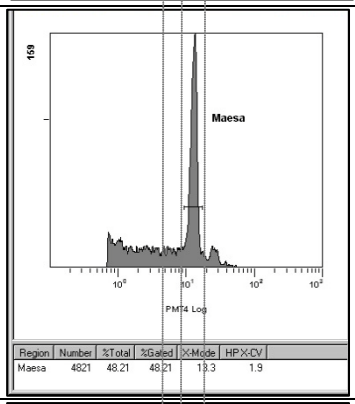
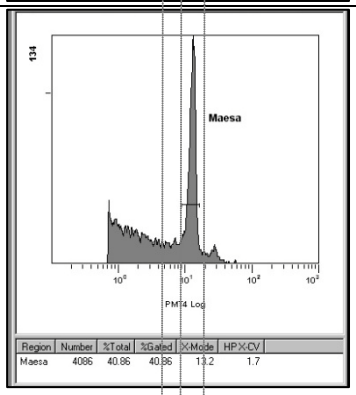
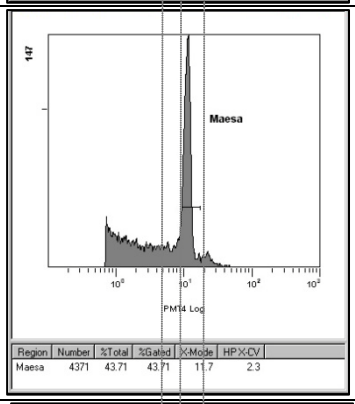
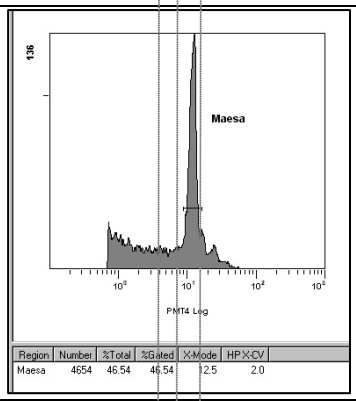
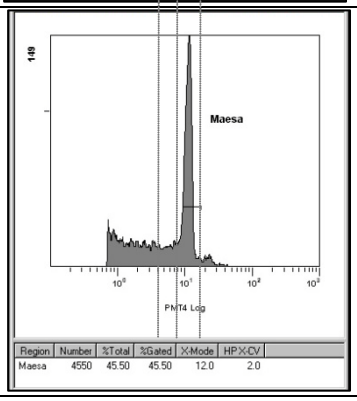
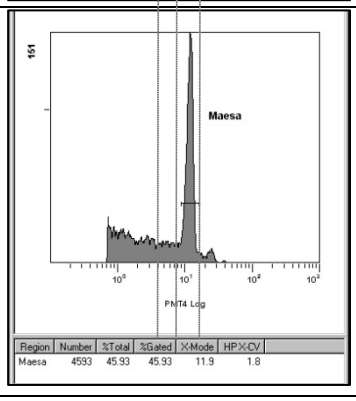
	M. argentea	*M. perlarius*
Greenhouse plants		
Regenerant 1		
Regenerant 2		
Regenerant 3		

Fig 3-7 Histograms of relative fluorescence intensity (log-transformed, PMT4 log) of isolated nuclei from *M. argentea* and *M. perlarius* shoots regenerated from callus compared to control greenhouse grown plants

Saponin analysis

Polyploid plants have been reported to produce higher amounts of secondary metabolites; therefore, the same plantlets that were analyzed for ploidy measurements were also used for saponin extraction and TLC analysis (Fig 3-7).

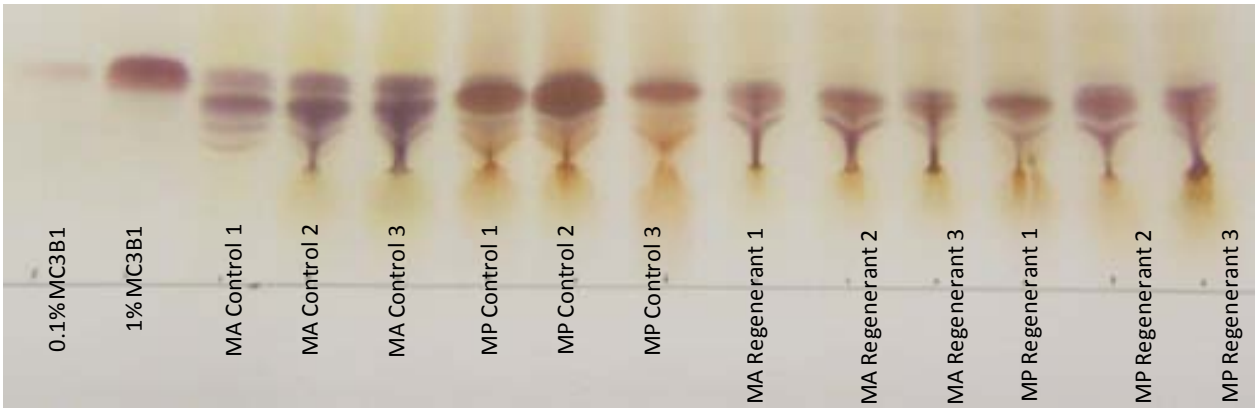


Fig 3-8 TLC analysis of saponin production in control *in vitro* plants and plantlets regenerated from callus for *M. argentea* (MA) and *M. perlarius* (MP). MC3B1 is an HPLC purified saponin mixture of *M. lanceolata* saponins and is used here as a reference sample 0,1% and 1% (w/v). Different repeats represent different individual plants.

Although *Maesa* calli does not produce detectable amounts of saponin (Chapter 4), plantlets regenerated from the same callus material synthesized a substantial amount of saponins, comparable to control plants. This indicated that differentiation was necessary for saponin production in *Maesa* species. *M. argentea* control plants had two major bands with retention factors (R_f) of 0.14 and 0.17 and also some minor bands. For regenerated *M. argentea* plantlets we could not clearly observe both bands; it seemed that only one band, with a R_f of about 0.16 was present. *M. perlarius* control plants produced only one major band with a R_f of 0.16. Regenerated plants showed a similar spot. Based on this TLC, the saponin content of polyploid plants does not seem to be higher than in control plants. Because the saponin content was found to be developmentally regulated and the plantlets analyzed here are still very young, further saponin analyses need to be performed on older greenhouse acclimatized plants before we can make solid conclusions on the saponin biosynthesis capacity of the polyploid plants.

Morphology of polyploid plants

Morphology of polyploid plants was macroscopically observed 3 months after transfer to the greenhouse (Fig 3-9). At this growth stage, the regenerated plants are smaller than the control plants. Also the leaves of the regenerated plants are smaller than those of the control plants. Further experiments are required to investigate the growth characteristics in different growing seasons and to monitor leaf morphology and development. In addition, we will determine cell size and perform DAPI nuclear staining.

Fig 3-9 *M. argentea* control (**a**) and polyploid regenerated (**b**) plants and *M. perlarius* control (**c**) and polyploid regenerated (**d**) plants. The plants have been acclimatized to greenhouse conditions and have been growing during 3 months *ex vitro*.

3.3.6 Adventitious shoot induction

A rapid protocol for shoot induction in *Maesa* would be interesting not only for micropropagation goals but also for future transformation experiments. Therefore, it was tested whether shoots could directly or indirectly be induced on isolated leaves of four *Maesa* species.

Shoot induction

To obtain adventitious shoot induction, leaf explants were incubated on MS medium supplemented with various concentrations of either BA or TDZ, individually or in combination with NAA. Adventitious shoots were observed for all four *Maesa* species; however, optimal shoot induction conditions differed for the analyzed species (Table 3-8). Generally, small outgrowths were induced at the surface of leaf explants after 4 to 5 weeks of culture. No shoots were induced on hormone-free medium or when cytokinins alone were added to the culture medium, implying that a combination of cytokinin and auxin was necessary for adventitious shoot formation in *Maesa* leaf explants. The only exception is shoot induction on *M. argentea* leaves with the highest concentration of TDZ.

Application of BA in combination with NAA resulted in very low frequency shoot formation for two out of the four *Maesa* species (Table 3-8). For *M. argentea*, shoot induction was only achieved with 22.2 µM BA and 1.35 µM NAA (0.2 shoots per explant). For *M. perlarius* two combinations of BA and NAA, namely 13.3 µM BA with 1.35 µM NAA and 22.2 µM BA with 2 µM NAA induced adventitious shoots, with a mean of 2.3 and 0.3 shoots per explant, respectively. For all four *Maesa* species, treatment of leaves with BA in combination with NAA often lead to root induction, which was not observed when BA alone was used.

In contrast to the results of shoot induction using BA in combination with NAA, the combination of TDZ and NAA induced multiple adventitious shoots on all four *Maesa* species (Table 3-8). For *M. argentea*, the highest average number of shoots (9.7 shoots per explant) was obtained when leaf explants were incubated on MS medium supplemented by 13.6 µM TDZ and 2 µM NAA (Fig 3-9). Except for the two lower concentrations of TDZ alone (4.5 and 13.6 µM), all the combinations and concentrations of TDZ and NAA resulted in shoot induction for *M. argentea*. For the other species shoots were also observed, however, not so frequently as for *M. argentea*. *M. lanceolata* leaves showed shoot induction with three combinations of TDZ and NAA, with a maximum of 4.1 shoots per explant when 22.7 µM TDZ was combined with 1.35 µM NAA (Fig 3-9). *M. perlarius* leaf explants developed adventitious shoots with only

one combination, 4.5 µM TDZ and 0.5 µM NAA, with a mean of 3.6 adventitious shoots per explant (Fig 3-9). *M. balansae* showed a lower frequency of shoots regeneration with at maximum 1.2 shoots per explant when the highest concentrations of TDZ and NAA were combined (Fig 3-9). A combination of 4.5 µM TDZ and 0.5 µM NAA also induced shoots, however, at a very low number (0.6 shoots per explant). Except for *M. argentea*, all species showed root formation in addition to shoot formation (Table 3-8).

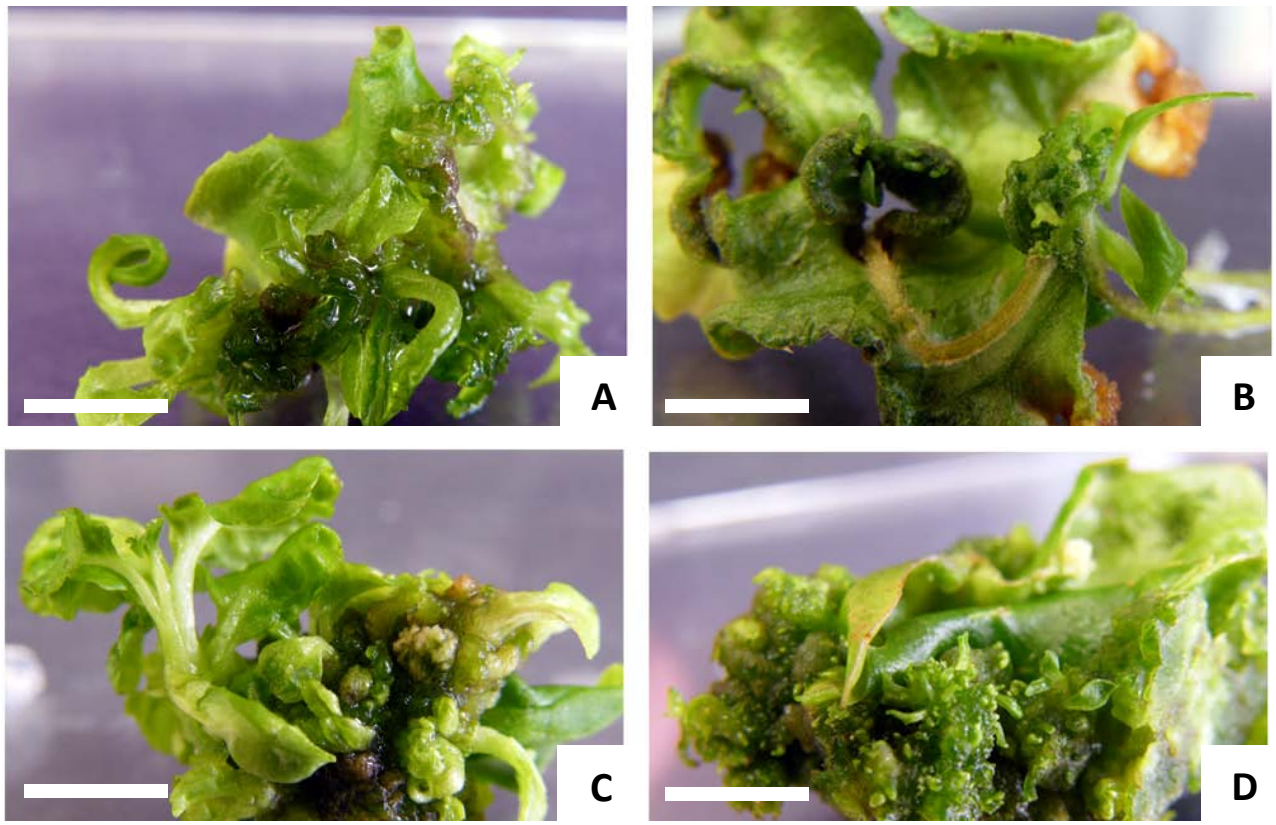


Fig 3-10 Induction of adventitious shoots on leaf explants of *M. argentea* (**a**), *M. balansae* (**b**), *M. lanceolata* (**c**) and *M. perlarius* (**d**). Pictures were taken 8 weeks after culture on MS medium supplemented with TDZ and NAA. Bar = 0.5 cm

Table 3-8 Effect of the plant growth regulators BA, TDZ and NAA on adventitious shoot regeneration from mature leaf explants of 4 *Maesa* spp. Shoot induction was evaluated and scored after 8 weeks;

Plant growth regulators (µM)			*M. argentea*		*M. balansae*		*M. lanceolata*		*M. perlarius*	
BA	TDZ	NAA	# shoots/explant (± SE)	Rooting	# shoots/explant (± SE)	Rooting	# shoots/explant (± SE)	Rooting	# shoots/explant (± SE)	Rooting
-	-	-	0.0 ^{c}	no	0.0 ^{a}	no	0.0 ^{b}	no	0.0 ^{b}	no
4.4	-	-	0.0 ^{c}	no	0.0 ^{a}	no	0.0 ^{b}	no	0.0 ^{b}	no
13.3	-	-	0.0 ^{c}	no	0.0 ^{a}	no	0.0 ^{b}	no	0.0 ^{b}	no
22.2	-	-	0.0 ^{c}	no	0.0 ^{a}	no	0.0 ^{b}	no	0.0 ^{b}	no
4.4	-	0.5	0.0 ^{c}	no	0.0 ^{a}	no	0.0 ^{b}	no	0.0 ^{b}	yes
4.4	-	1.35	0.0 ^{c}	no	0.0 ^{a}	yes	0.0 ^{b}	yes	0.0 ^{b}	yes
4.4	-	2	0.0 ^{c}	no	0.0 ^{a}	yes	0.0 ^{b}	yes	0.0 ^{b}	yes
13.3	-	0.5	0.0 ^{c}	no	0.0 ^{a}	yes	0.0 ^{b}	no	0.0 ^{b}	yes
13.3	-	1.35	0.0 ^{c}	yes	0.0 ^{a}	yes	0.0 ^{b}	yes	2.3 ± 0.85 ^{a}	yes
13.3	-	2	0.0 ^{c}	yes	0.0 ^{a}	yes	0.0 ^{b}	yes	0.0 ^{b}	yes
22.2	-	0.5	0.0 ^{c}	no	0.0 ^{a}	yes	0.0 ^{b}	no	0.0 ^{b}	no
22.2	-	1.35	0.2 ± 0.22 ^{c}	yes	0.0 ^{a}	yes	0.0 ^{b}	no	0.0 ^{b}	no
22.2	-	2	0.0 ^{c}	yes	0.0 ^{a}	yes	0.0 ^{b}	yes	0.3 ± 0.33 ^{b}	no
-	4.5	-	0.0 ^{c}	no	0.0 ^{a}	no	0.0 ^{b}	no	0.0 ^{b}	no
-	13.6	-	0.0 ^{c}	no	0.0 ^{a}	no	0.0 ^{b}	no	0.0 ^{b}	no
-	22.7	-	1.7 ± 0.85 ^{bc}	no	0.0 ^{a}	no	0.0 ^{b}	no	0.0 ^{b}	no
-	4.5	0.5	0.4 ± 0.34 ^{c}	no	0.6 ± 0.34 ^{a}	yes	0.0 ^{b}	no	**3.6 ± 0.62** ^{a}	no
-	4.5	1.35	4.9 ± 2.98 ^{b}	no	0.0 ^{a}	yes	0.0 ^{b}	no	0.0 ^{b}	no
-	4.5	2	0.4 ± 0.44 ^{c}	no	0.0 ^{a}	yes	0.0 ^{b}	yes	0.0 ^{b}	yes
-	13.6	0.5	0.7 ± 0.37 ^{c}	no	0.0 ^{a}	yes	0.0 ^{b}	no	0.0 ^{b}	no
-	13.6	1.35	5.0 ± 1.24 ^{b}	no	0.0 ^{a}	yes	1.3 ± 0.57 ^{ab}	no	0.0 ^{b}	no
-	13.6	2	**9.7 ± 2.06** ^{a}	no	0.0 ^{a}	yes	0.0 ^{b}	yes	0.0 ^{b}	yes
-	22.7	0.5	5.4 ± 0.93 ^{b}	no	0.0 ^{a}	yes	0.0 ^{b}	no	0.0 ^{b}	no
-	22.7	1.35	2.6 ± 0.94 ^{bc}	no	0.0 ^{a}	yes	**4.1 ± 1.58** ^{a}	no	0.0 ^{b}	no
-	22.7	2	1.6 ± 0.84 ^{bc}	no	**1.2 ± 1.2** ^{a}	yes	1.9 ± 1.2 ^{ab}	yes	0.0 ^{b}	yes

Different letters indicate significant differences ($p<0.05$) within one species according to Tukey test

Rooting and acclimatization

Regenerated shoots elongated and developed roots in basal MS media without phytohormones with an efficiency of 100% (Table 3-9). Generally, *Maesa* roots started to emerge within 2 weeks. Plantlets spontaneously produced well developed root system within 6 weeks on hormone-free medium (Fig 3-10). The very efficient rooting mechanism of these plants provides an additional advantage for the rapid clonal propagation without any growth regulators needed. This spontaneous root formation is effective during the establishment of the plantlets in soil as well. For acclimatization, regenerated and rooted shoots from all *Maesa* spp. could be hardened with a 100% efficiency rate (Table 3-9). After transfer to greenhouse condition, the plantlets continued to grow and developed into normal and vigorous plants (Fig 3-10).

Table 3-9 Rooting and acclimatization of shoots derived from axillary bud multiplication and adventitious shoots from leaf explants

Sample		Rooting		Acclimatization	
Species	Explant type	# samples	% rooting	# samples	% acclimatization
M. argentea	Adventitious shoots	25	100	10	100
M. balansae	Adventitious shoots	11	100	5	100
M. lanceolata	Adventitious shoots	17	100	10	100
M. perlarius	Adventitious shoots	12	100	5	100

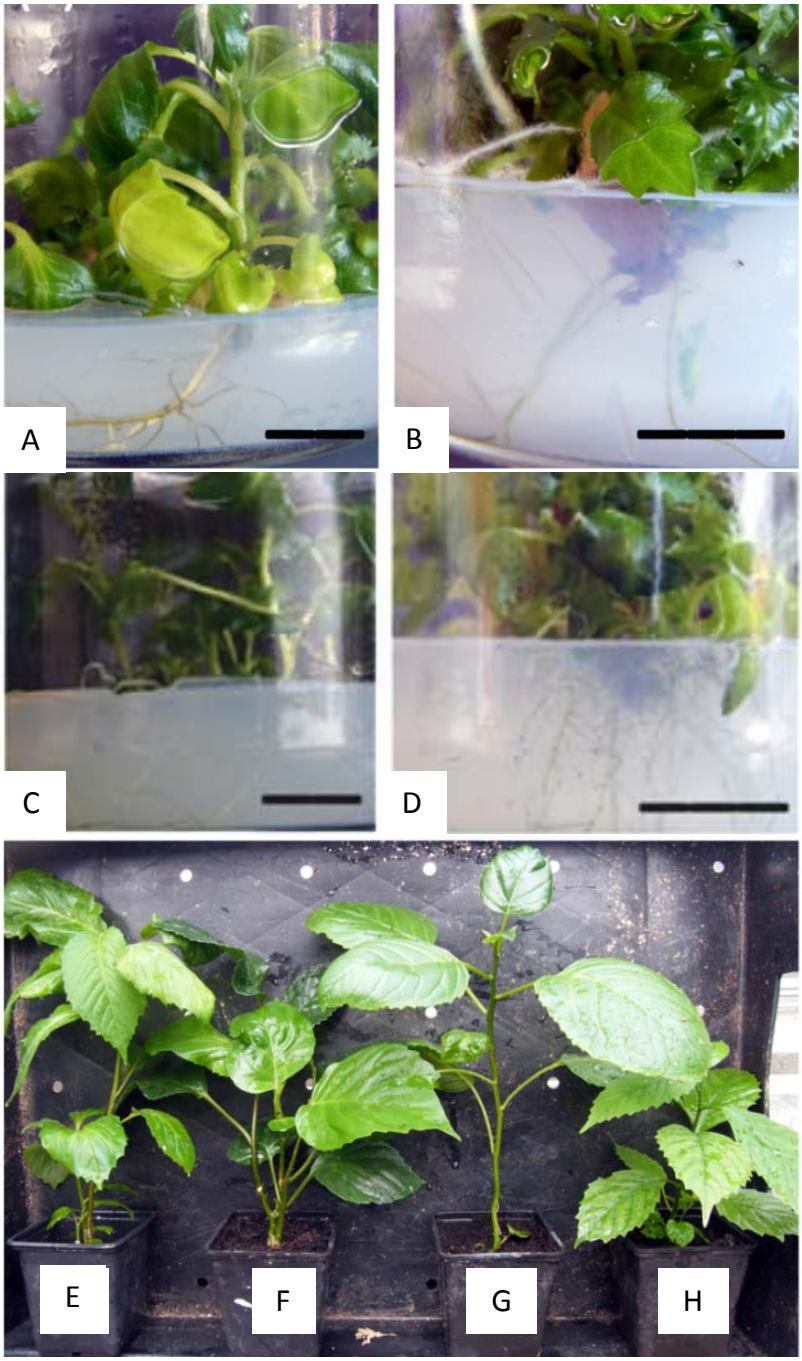


Fig 3-10 Rooting of *in vitro* regenerated shoots of *M. argentea* (**a**), *M. balansae* (**b**), *M. lanceolata* (**c**) and *M. perlarius* (**d**). Root pictures were taken 8 weeks after culture on MS basal medium. Regenerated shoot acclimatized for 4 months in greenhouse conditions of *M. argentea* (**e**), *M. balansae* (**f**), *M. lanceolata* (**g**) and *M. perlarius* (**h**). Bar = 1 cm

Ploidy analysis

Maesa plantlets regenerated through adventitious shoot induction were also subjected to flow cytometric analysis. Results for ploidy measurements are represented in Figure 3-11.

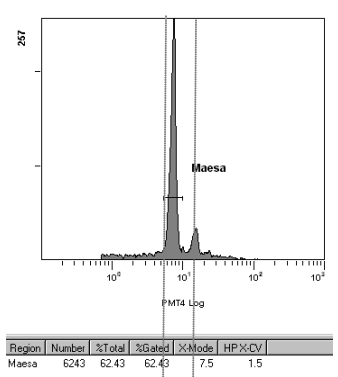
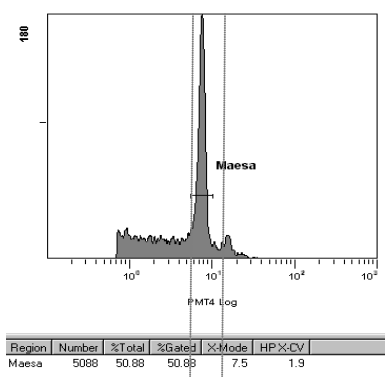
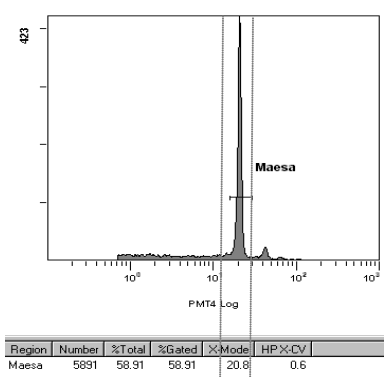
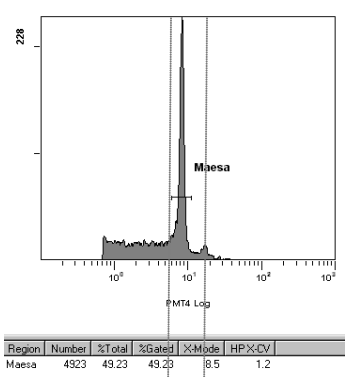
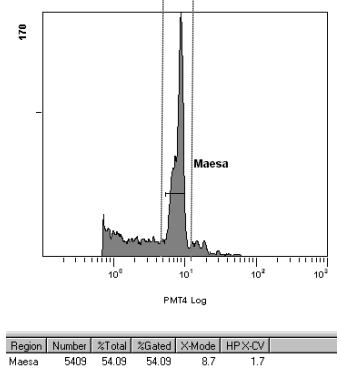
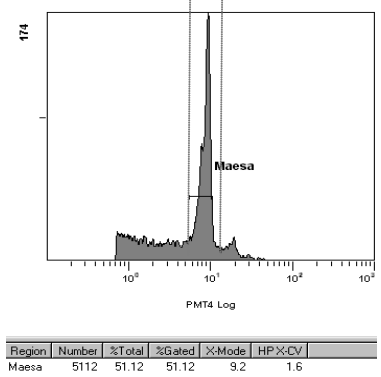
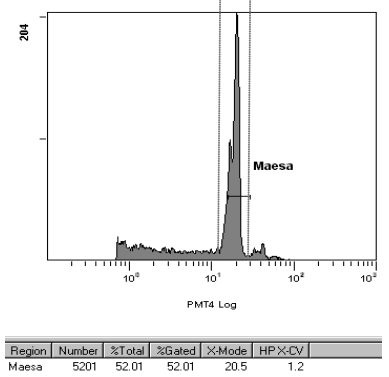
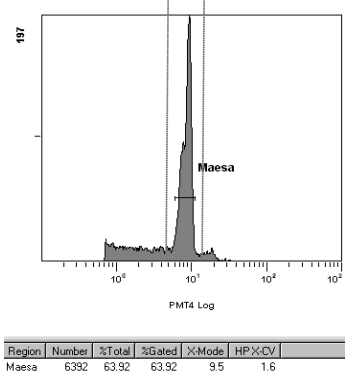
	Maesa argentea	*Maesa balansae*	*Maesa lanceolata*	*Maesa perlarius*
Greenhouse plants				
Adventitious shoots				

Fig 3-12 Histograms of relative fluorescence intensity (log-transformed, PMT4 log) of isolated nuclei from four *Maesa* species regenerated through axillary shoot multiplication compared to control greenhouse grown plants

The flow cytometry analysis showed similar peaks in all samples within every *Maesa* species (Fig 3-11). No significant differences in DNA content were observed for each of the *in vitro* cultivated *Maesa* plant material indicating that no changes in ploidy level occurred during the regeneration process. In addition, early stage hormone stimulated leaf explants was also analyzed to check for ploidy level changes. Also in this plant material we did not observe deviations in DNA content, indicating that the hormone treatment did not alter the ploidy level of the plants (results not shown).

Saponin analysis

Using purified *M. lanceolata* saponins (sample MC3B1) as a reference standard, qualitative TLC analysis showed that regenerated shoots produced a phytochemical profile similar to shoots of greenhouse plants. Saponins from *M. lanceolata* was characterized by the appearance of single spot at R_f = 0.17. *Maesa balansae* and *M. perlarius* produced a similar profile consisting of a major spot with R_f = 0.16 and a series of minor spots with lower R_f values. These minor compounds were not observed in the *M. lanceolata* extracts. *M. argentea* showed a different profile with two major compounds with R_f = 0.14 and 0.17.

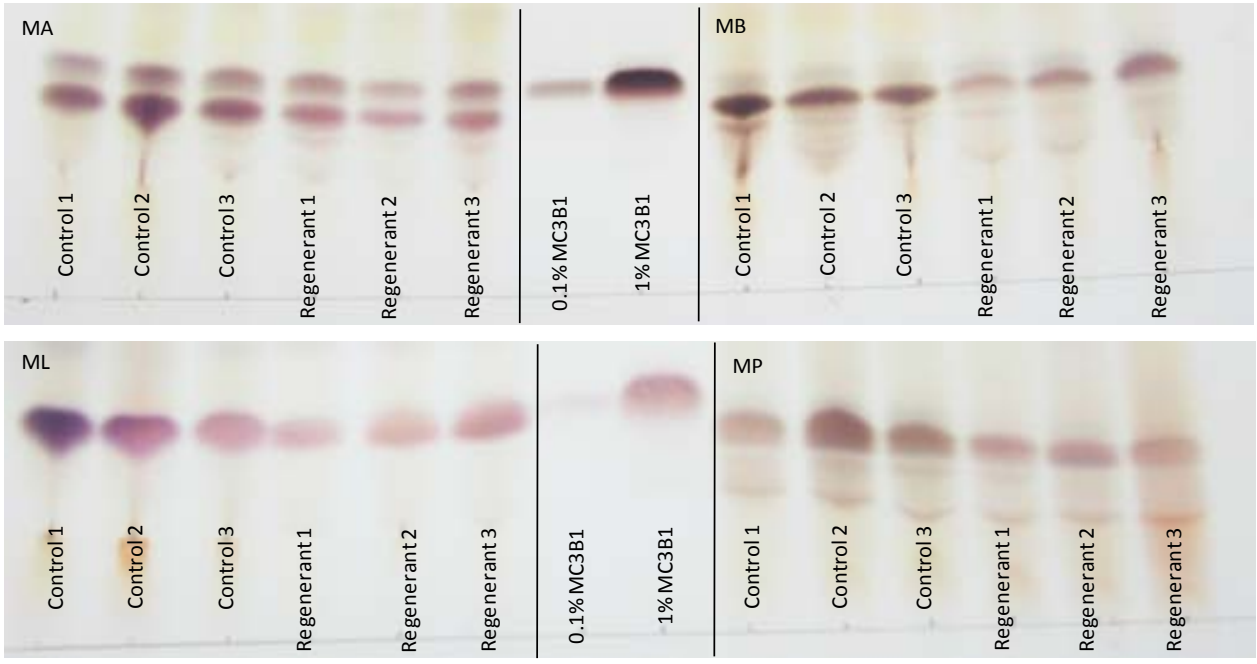


Fig 3-13 TLC analysis of saponin production in control *in vitro* plants and plantlets induced through adventitious shoots induction for *M. argentea* (MA), *M. balansae* (MB), *M. lanceolata* (ML) and *M. perlarius* (MP). MC3B1 is an HPLC purified saponin mixture of *M. lanceolata* saponins and is used here as a reference sample 0.1% and 1% (w/v). Different repeats represent different individual plants.

3.4 Discussion

Pharmaceutical companies largely depend upon phytochemicals obtained from naturally occurring stands with the consequence that these natural supplies are rapidly being depleted. Many valuable medicinal plants are already under the threat of extinction. In addition, chemical synthesis is economically not feasible for highly complex molecules (or mixtures) or has not yet been achieved for most of the medicinal compounds derived from plants. In this respect, plant tissue culture could be an alternative method for commercial propagation (Rout et al. 2000). The culture of plant cells and tissues on a large scale was even seen as a more convenient and reliable source of secondary products than intact plants. Cell and tissue culture does offer the manufacturer independence from fluctuations in supply of the raw plant material (Collin 2001). For *Maesa* spp., cell and tissue cultures were especially important because seeds were collected from nature and it is not clear how variable this material really is.

3.4.1 Callus cultures and cell suspensions

Callus culture on solid medium is the first step to develop a liquid culture as a practical source for scaling up and engineering secondary metabolite production. Calli are also convenient for the maintenance of cell lines and are often the form of culture from which plant regeneration is initiated. One of the major success stories in production of saponins in cell suspensions is ginseng cell culture. Field cultivation of ginseng takes about 5 – 7 years from seedling to final harvest and during that period plants are subjected to climate changes, pests and pathogens. Therefore cell cultures were considered as a potential alternative. In fact, ginseng cell culture has been applied commercially in Japan by the Nitto Denko Corporation, at volumes up to 20 000 and 25 000 L achieving a biomass productivity of 714 mg/L per day (Wu and Zhong 1999). The chemotherapeutic agent taxol is currently also supplied through both a semi-synthetic process and plant cell culture (Vongpaseuth and Roberts 2007).

For *Maesa lanceolata* and *M. perlarius*, the auxin 2,4-D alone was sufficient to induce callus on leaf explants. For *M. argentea* a combination of 2,4-D with cytokinin was necessary for callus induction and for *M. balansae* callus was established when using a combination of 2,4-D with BA, kinetin or NAA. The phytohormone 2,4-D was also effective for callus induction in the saponin producing plants *Helicteres isora* and *Allium chinense* (Shriram et al. 2008; Yan et al. 2009). *Maesa* callus showed an exponential growth on solid medium. A similar growth curve was found for calli of the flavonoid producing plant

Cytisus aeolicus on medium with 2,4-D and kinetin, supplemented with myo-inositol (Lucchesini et al. 2010). Small scale *M. lanceolata* batch cell cultures were also initiated. Three growth phases were distinguished: a lag phase, an exponential phase and a stationary phase. The exponential growth phase itself could be divided into three "sub"phases; at onset the cells divided rapidly, followed by a phase in which cell number increased more or less linearly and finally a progressive deceleration was noticed. Not all of the cell suspensions showed a clearly defined five-phase growth (Thomas and Davey 1975). *M. lanceolata* cell suspensions had a clear lag phase during the first week after subculturing. Next, an exponential and a linear phase were noticed between day 7 and 11. Finally, in the last days before subculture the packed cell volume still increased but at a slower rate compared to the exponential phase. This last growth period is probably the start of the deceleration phase. Cells were subcultured after two weeks, before a stationary phase was reached, because growth periods of longer than two weeks resulted in browning of the cultures. The phenomenon of 'culture browning' was, however, also observed for some cultures shortly after subculturing. Preliminary tests showed that this was not due to the density of the cultures, negative factors in the culture medium or mechanical damage of the cells during subculturing. Culture browning was also observed for *Glycyrrhiza inflata* cell suspensions in a bioreactor and this was overcome by application of a continuous perfusion culture system (Wang and Qi 2009). The problem of tissue browning is also reported for callus cultures. For example, callus cultures from *Pinus sylvestris* were characterized by rapid browning. Results suggested that early browning was correlated with high peroxidase activities (Laukkanen et al. 1999). In *Panax ginseng* cell cultures, ultrasound-induced stress resulted in higher polyphenol oxidase activity and higher rate of polyphenol production. This resulted in enzymatic browning of the cultures (Wu and Lin 2002). Further research will, however, be necessary to find the cause and solution for culture browning in cell suspensions of *M. lanceolata*.

3.4.2 Methods for *in vitro* propagation

Organogenesis and regeneration techniques for *Maesa* species could not only play an important role in germplasm conservation, though also in genetic and metabolic improvement of these plants. For *in vitro* propagation of *M. argentea, M. balansae, M. lanceolata* and *M. perlarius* three different protocols were developed.

A protocol for **axillary shoot formation** was successful for all four species. For *M. argentea, M. balansae* and *M. lanceolata* the number of shoots per explant was always highest when treated with BA alone.

Addition of NAA had a negative effect on the number of shoots formed. This is in contrast with the results published for *Maesa ramentacea*, where a synergistic action between BA and NAA was observed (Kanchanapoom and Boonvanno 2000). For *M. perlarius* none of the treatments gave significantly more shoots compared to the control plantlets. Noticeably, the multiplication rate of the control plants of *M. perlarius* was higher than that of the controls of the other species. Similar axillary shoot induction protocols were also developed for the medicinal plants *Searsia dentata* (Prakash and Van Staden 2008), *Asparagus racemosus* (Bopana and Saxena 2008), *Cytisus aeolicus* (Lucchesini et al. 2010), *Clinoria ternatea* (Pandeya et al. 2010), *Balanites aegyptiaca* (Anis et al. 2010) and *Jatropha curcas* (Kaewpoo and Te-chato 2009). In all these protocols, BA proved to be the most important cytokinin for multiple shoot induction, sometimes in combination with auxin or with another cytokinin.

The **regeneration of callus** material to plants has also successfully been used for mass propagation and for obtaining useful variants in several medicinal plant species (Rout et al. 2000). Callus of *M. argentea, M. balansae* and *M. perlarius*, cultured during 1 year, maintained competence to form shoots. 15 different combinations of BA and kinetin were tested for callus regeneration; however, none of these hormone combinations was effective in inducing shoots. In contrast, a combination of TDZ and NAA was effective in shoot induction in three out of the four species. Remarkably, *M. argentea* callus formed the highest number of shoots on basal medium without any hormones. So for this species, it appeared that addition of phytohormones repressed shoot formation instead of stimulating it. Callus regeneration protocols have been described for many other medicinal plants and effective hormone combinations were clearly dependent on the plant species used. For example, a combination of BA and kinetin was most effective for callus regeneration of *Helicteres isora* (Shriram et al. 2008), BA and TDZ for *Psoralea corylifolia* (Baskaran and Jayabalan 2009), kinetin and NAA for *Lonicera macranthoides* (Wang et al. 2009) and BA and NAA for *Allium chinense* (Yan et al. 2009).

A third method for micropropagation of *Maesa* species was based on **adventitious shoot induction** on leaves. Culture medium comprising BA and NAA was not very efficient and induced shoots only on *M. argentea* and *M. perlarius* leaves. In contrast, NAA in combination with TDZ lead to shoot induction for all four species. Shoots were not induced on hormone-free medium or when cytokinins alone were added to the culture medium, implying that a combination of cytokinin and auxin was necessary for adventitious shoot formation in *Maesa* leaf explants. The only exception is shoot induction on *M. argentea* leaves with the highest concentration of TDZ. On the contrary, BA is the most efficient phytohormone for shoot induction for many plants, e.g. for *Asparagus cochinchinensis* (Jiang et al. 2010),

Chamaecyparis obtura (Min et al. 2010), *Lycopersicon esculeutum* (Mohamed et al. 2010), *Citrus sinensis* (Singh and Rajam 2010) and *Erigeron breviscapus* (Xing et al. 2010).

Taken together, the results of callus regeneration and adventitious shoot induction suggest that TDZ acts synergistically with NAA to promote shoot induction in *Maesa*. In contrast to addition of BA and NAA or BA and kinetin, TDZ played an essential role in inducing adventitious shoots on leaf explants and callus and proved to be the more effective cytokinin in our study. The higher effectiveness of TDZ as compared to BA, was reported for some plant species such as *Mimulus aurantiacus* (Murovec et al. 2010), *Lysimachia* spp. (Zheng et al. 2009), *Paulownia tomentosa* (Corredoira et al. 2008) and *Echinacea purpurea* (Jones et al. 2007). TDZ, a synthetic phenylurea derivative, is one of the most active cytokinin-like compounds for woody plant tissue culture (Huetteman and Preece 1993; Lu 1993). Unlike classic cytokinins, TDZ is competent of fulfilling both the cytokinin and auxin requirement of various regenerative responses of many different plant species (Jones et al. 2007). A low concentration of NAA was necessary to induce direct shoot regeneration from leaf explants. This means that NAA can be considered as a critical growth regulator for shoot regeneration of *Maesa* spp. De Gyves and coworkers hypothesized that there is a synergism existing between TDZ and both endogenous and exogenous auxin (De Gyves et al. 2001). This finding corresponds with our results that in general the combination of TDZ and NAA promoted more shoots compared to application of TDZ alone. The combination of TDZ and NAA has also been reported to induce shoot regeneration from leaf explants of several plant species (Espinosa et al. 2006; Zhou et al. 2010).

3.4.3 Rooting and acclimatization of regenerated shoots

Shoots that were obtained with all three of the above described propagation protocols elongated and developed roots in basal MS media without phytohormones with a very high efficiency. Generally, plantlets spontaneously formed a well developed root system within 6 – 8 weeks on hormone-free medium. The very efficient rooting mechanism of these plants provides an additional advantage for the rapid clonal propagation without any growth regulators needed. This spontaneous root formation is effective during the establishment of the plantlets in soil as well. Rooting of shoots without any addition of hormones is also reported for *Vitis champinii* (Mukherjee et al. 2010), *Tuberaria major* (Gonçalves et al. 2010), *Drymaria cordata* (Ghimire et al. 2010) and *Aloe polyphylla* (Bairu et al. 2007). However, most species require auxin treatment for root induction prior to acclimatization.

3.4.4 Genetic and biochemical characteristics of regenerated shoots

Inducing adventitious shoots through *in vitro* regeneration could be accompanied by genetic instability through a process known as somaclonal variation (Larkin and Scowcroft 1981). Since this regeneration process escapes the normal plant fertilization and development, it is also possible that the variation occurs by epigenetic factors (Kaeppler et al. 2000). The variability that is commonly noticed are the ploidy level, chromosome structure, mitotic abnormalities and other cytological disorders (Radić et al. 2005). Flow cytometry is considered as a powerful tool for estimating DNA ploidy level in plant *in vitro* (Cousin et al. 2009) and has been used to investigate the genetic variability of *in vitro* regenerated shoots (Mallón et al. 2010; Vujovic et al. 2010). The relative nuclear DNA content of *Maesa* spp. regenerated through axillary and adventitious shoot formation did not differ significantly from control greenhouse plants grown from seedlings, indicating that no changes in ploidy level occurred during the regeneration process. These results are in line with genetic analysis of adventitious and axillary shoots of *Olea europeae* and *Olea maderensis* (Brito et al. 2010), *Centaurea ultreiae* (Mallón et al. 2010) and *Rubus fruticosus* (Vujovic et al. 2010). In contrast, *M. argentea* and *M. perlarius* plantlets regenerated from callus material did show polyploidization. Also the original callus material was polyploid. Polyploidization of callus material and plants regenerated from callus has been described for *Curcuma aromatica* (Mohanty et al. 2008), *Beta vulgaris* (Weber et al. 2010), *Camellia sinensis* (Rout et al. 1998) and *Tricyrtis hirta* (Nakano et al. 2006).

Because we are interested in the saponins produced by *Maesa* species, it was important to assess the capacity to produce saponin in *in vitro* regenerated plants. TLC analysis revealed that adventitious and axillary induced shoots showed a phytochemical profile similar to shoots of greenhouse plants. Although the saponin detection method applied here does not allow a solid assessment of the amount of saponin produced, we are confident that the *in vitro* regenerated shoots had maintained a significant capacity to produce and accumulate saponins.

Polyploid plants often are superior to diploids with respect to morphological changes, genetic adaptability and tolerance to environmental stresses (Dhooghe et al. 2010; Xiong et al. 2006). Certain polyploids also produce more secondary metabolites than the corresponding diploids (Berkov and Philipov 2002; Gao et al. 2002; Gao et al. 1996), however, polyploid plants regenerated from *M. argentea* and *M. perlarius* calli did not seem to have a higher saponin content than control plants. Saponin analyses of the callus material used for regeneration revealed that calli themselves are not capable of producing detectable amounts of saponins (Chapter 4). The incompetence of undifferentiated tissues to

produce secondary metabolites is a generally observed phenomenon (Bourgaud et al. 2001; Collin 2001; Wink 1989). Yet, plants regenerated from these calli do produce saponins; hence, we can conclude that differentiation is necessary for saponin production in *Maesa* species. Similar observations were also made for *Bupleurum falcatum*: *B. falcatum* callus did not produce saponins but in roots growing from the callus saponins were again detected (Yamamoto and Kamura 1997).

3.4.5 Conclusion

In conclusion, we have established different types of *in vitro* cultures for four *Maesa* species. Calli and *in vitro* plantlets will be further analyzed for saponin production (Chapter 4) and were used as explant material for hairy root induction and protoplast isolation (Chapter 5 and 7). In addition, we have developed three protocols for micropropagation of *Maesa in vitro* plantlets. Axillary and adventitious shoot induction protocols were efficient for all *Maesa* species and produced "true to type" *Maesa* plants in terms of ploidy and saponin production. Regeneration of callus material to plants was only successful for *M. argentea* and *M. perlarius* and resulted in polyploids plants. Polyploidization did not have an effect on saponin production, though; differentiation did prove to be necessary for efficient saponin synthesis.

3.5 Materials and methods

3.5.1 Plant Material

M. argentea, M. balansae, M. lanceolata and *M. perlarius* seeds were purchased as mentioned in the Materials and methods section of Chapter 2. Protocols for *in vitro* initiation and culture were also described in the Materials and methods section of Chapter 2.

3.5.2 Ploidy analysis: flow cytometry

Flow cytometry was performed to check ploidy of regenerated shoots. Leaf samples derived from micropropagation through axillary branching, callus regeneration and adventitious shoot induction were compared with greenhouse grown adult plants. Approximately 50-100 mg of both young *in vitro* and wild adult plant leaf material was chopped with a razor blade in 2 ml Galbraith buffer (45 mM $MgCl_2$ (VEL), 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS) (Fluka), 30 mM sodium citrate (VEL) and 0.1% (v/v) Triton X-100 (Sigma)) (Galbraith et al. 1983; Loureiro et al. 2006) to isolate nuclei. 5% (w/v) of Polyvinylpyrrolidone 1000 (PVP-10) (Sigma) was also added to the extraction buffer to neutralize interference of cell metabolites in the measurements. The nuclei suspension was filtered through a 50 µm strainer to remove debris (BD Biosciences Europe). 400 µl of nuclei suspension was then mixed with 50 µl propidium iodide (0.5 mg/ml) (ICN) and 50 µg/ml RNase (Roche). The DNA content of the isolated samples was analyzed using a Beckman Coulter EPICS® *ALTRA*™ Flow Cytometer. The instrument was equipped with a 15 mW 488 nm air-cooled argon-ion laser. Fluorescence was detected through a 575 nm band-pass filter. Disintegrated nuclei and other cell debris signals were eliminated from analysis by two gating systems; forward scatter based on nuclei proportional and PMT3 based on propidium iodide fluorescence. The gates were consistently maintained for all samples in each run and the resulting PMT4 histograms were analyzed using EXPO™32 MultiCOMP software (Beckman Coulter). Together with each leaf sample, leaf tissue from diploid (2n = 2x) *Arabidopsis thaliana* was included as an external reference standard.

3.5.3 Biochemical analysis: thin layer chromatography

For TLC analysis, saponins were extracted as described in the Materials and methods section of Chapter 2. The protocol for TLC analysis of saponins is also described in the Material and methods section of Chapter 2.

3.5.4 Axillary shoot formation

Three month old seedlings of *Maesa argentea, M. balansae, M. lanceolata and M. perlarius* were used as explant source for micropropagation through an axillary branching method. Shoots were excised, defoliated and implanted vertically into MS basal medium supplemented with 3% (w/v) sucrose and 0.15% (w/v) Gelrite (Marck & Co., Kelco Division, USA) in a Meli-jar (4 shoots/jar) (De Proft et al. 1985).

For shoot multiplication, the basal medium was supplemented with 6-benzyladenine (BA) (Sigma) and 1-naphthaleneacetic acid (NAA) (BDH) at different concentrations. The following concentrations were used; 4.4 µM, 8.8 µM, 13.2 µM and 22.2 µM **BA** and 5 µM, 10.7 µM and 13.5 µM **NAA**. Shoots were incubated at 26°C in a 16/8h light/dark period and axillary shoot formation was evaluated 8 weeks later (Table 3-1). This experiment was performed in triplicate with 4 shoots per replicate.

3.5.5 Callus induction and growth

Leaves of *in vitro* grown *Maesa lanceolata*, *Maesa argentea, Maesa perlarius* and *Maesa balansae* were used as explants for callus induction. The leaves were isolated and cut into slices of +/- 0.5 cm thickness and were then put on MS basal medium supplemented with 0.8% (w/v) agar and 3% (w/v) sucrose (with pH 5.8). For callus induction auxins and cytokinins were added to the basal medium in different combinations and the effect of different hormones and combinations of hormones on callus induction was studied. The following growth regulators were used in the given concentration; 2.7 µM **NAA** (BDH), 4.4 µM **BA** (Sigma), 5 µM 2**,**4-dichlorophenoxy acetic acid (**2,4-D**) (Sigma) and 0.46 µM **kinetin** (Duchefa) (table 3-3). Leaf material was incubated at 25°C in the dark on Petri dishes (3 leaf slices/dish). The experiment was performed in triplicate with nine leaf slices per repeat. Callus formation was scored 6 weeks later. After evaluation, callus was put on fresh medium with the same composition as for the callus induction. Calli were subcultured every month. To establish a growth curve, the callus material on the different media was weighed every week (3 calli/medium).

3.5.6 Establishment and maintenance of cell suspensions

Cell cultures were established for *M. lanceolata* only. 200 – 300 mg of friable callus, 1 week post subculturing on medium with 5 µM 2,4-D and 0.46 µM kinetin, was suspended in 20 ml liquid MS medium in an 100 ml Erlenmeyer. The liquid medium was supplemented with 3% (w/v) sucrose, 5 µM **2,4-D** (Sigma) and 0.46 µM **kinetin** (Ducheffa). The cell suspensions were subcultured every two weeks. For this purpose one volume of the cell suspension was resuspended in three volumes of liquid medium. Cultures were grown in the dark at 25°C on a rotary shaker (120 rpm). For establishment of a growth curve, the cell suspensions were transferred to a 15 ml falcon tube and centrifuged in a swinging bucket centrifuge for 10 minutes at 300 rpm (Heraeus® Biofuge® Primo R centrifuge). The volume of cells in the suspension was registered and the percentage of cell volume in the suspension was calculated.

3.5.7 Callus regeneration

For regeneration, one year old callus of *Maesa argentea*, *M. balansae, M. lanceolata* and *M. perlarius* was used. The callus of *M. argentea* was grown on medium with 5 µM 2,4-D and 4.4 µM BA, callus of *M. balansae* and *M. lanceolata* was cultured on medium with 5 µM 2,4-D and 0.46 µM kinetin and finally callus of *M. perlarius* was grown on medium with only 5 µM 2,4-D. For regeneration, calli were transferred to Petri dishes with MS basal medium supplemented with 0.8% (w/v) agar and 3% (w/v) sucrose and different concentrations of hormones, either alone or in combinations (9 calli/dish). The

following phytohormones were used in the given concentrations; 0.44 µM, 2.22 µM and 4.44 µM **BA** (Sigma) and 0.46 µM, 2.32 µM and 4.64 µM **kinetin** (Duchefa) (Table 3-4). Callus on regeneration medium was incubated at 26°C with a 16/8h light/dark photoperiod. Six week after incubation on medium with BA and kinetin, the calli were split in two; half of the callus was placed on fresh medium with the same concentration of BA and kinetin and the other half was placed on medium with 4.5 µM **TDZ** (Duchefa) and 0.05 µM **NAA** (BDH). The experiment was performed in triplicate with nine calli per replicate. Shoot induction was scored 17 after the start of the experiment.

3.5.8 Chlorophyll measurements

For chlorophyll determination, 150 mg of fresh callus material was ground in liquid nitrogen and subsequently transferred to a 15 ml falcon tube covered with aluminum foil. 10ml of 80% (v/v) acetone (Chem-Lab NV) was added and the tubes were placed at -20°C for 24 hours. After incubation, 200µl of the samples were transferred to a 96-well plate (Corning Inc). Absorbance was measured at 663.2 and 646.8 nm with an Infinite® M200 plate reader (Tecan Group Ltd.) and analyzed with Magellan™ V6.6 software (Tecan Group Ltd). The chlorophyll a, chlorophyll b and total chlorophyll content were calculated as follows:

$\mathbf{C_a} = (12.25 \times 0.55 \times A_{663.2}) - (2.55 \times 0.55 \times A_{646.8})$
$\mathbf{C_b} = (20.31 \times 0.55 \times A_{646.8}) - (4.91 \times 0.55 \times A_{663.2})$
$\mathbf{C_T} = C_a + C_b$ (in µg/ml)

With $A_{663.2}$ = absorbance at 663.2 nm
$A_{646.8}$ = absorbance at 646.8 nm

3.5.9 Adventitious shoot induction

Fully developed leaves from *in vitro* grown plants of *Maesa argentea, M. balansae, M. lanceolata* and *M. perlarius* were isolated and used as explants. Leaves (petiole was cut off from the leaf bases) were placed in a Petri dish with the adaxial side in contact with MS basal medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar (3 leaves/dish). For shoot induction, auxin and cytokinins were added to the basal medium in different concentrations, either alone or in combinations. The following growth regulators were used in the given concentrations; 0.5 µM, 1.35 µM and 2 µM **NAA** (BDH); 4.4 µM, 13.3 µM and 22.2 µM **BA** (Sigma) and 4.5µM, 13.6 µM and 22.7 µM **TDZ** (Duchefa) (Table 3-7). Leaves were incubated at 26°C with 16/8h light/dark photoperiod. The average number of adventitious shoot induced per explant was recorded after 8 weeks of culture. The experiment was performed in triplicate with three leaves per replicate.

3.5.10 Rooting and acclimatization

Multiple shoots were formed through axillary branching, callus regeneration and adventitious shoot induction. These shoots were isolated and transferred to basal MS medium lacking growth regulators

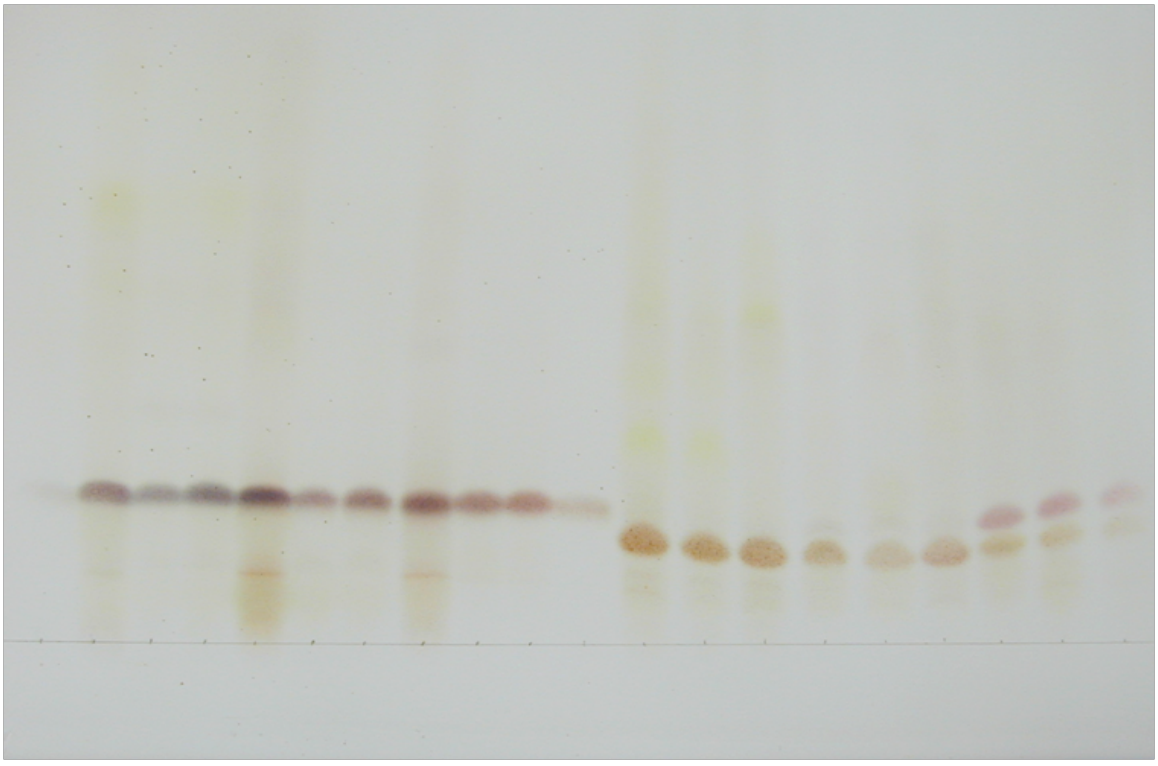
and supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar, for elongation and rooting in one single step. Shoots were incubated in a 16/8h light/dark photoperiod at 25°C.

Rooted plantlets were gently and thoroughly washed with water, to remove attached medium from the roots, and were transferred to 9 x 9 cm^2 small plastic pots containing a mixture of sand and peat soil (1:1) (Groep AVEVE). The plantlets were placed in a small greenhouse with a high humidity for 3 weeks to gradually acclimatize to greenhouse conditions.

3.5.11 Statistical analysis

Each petri dish and bottle with three to five explants was considered as one replicate. The presented experiments are comprised of at least three replicates. Number of shoots was recorded 8 weeks after induction both for axillary shoot formation and adventitious shoot from leaf explants and 17 weeks after callus regeneration. The data were analyzed by one-way ANOVA followed by Tukey test ($P<0.05$).

CHAPTER 4 SAPONIN PRODUCTION IN MAESA IN VITRO CULTURES



4.1 Abstract

In vitro cultures are often used as a stable plant source for the production and analysis of secondary metabolites. Depending on the culturing conditions and the plant species, the production levels can vary which impedes a biochemical characterization. Therefore, it is important to investigate the properties of the *in vitro* cultures established in Chapter 3. We analysed the saponin content in *in vitro* shoot cultures, callus and hairy roots and compared this with the saponin content in greenhouse grown plants. The saponin content of *Maesa argentea*, *M. balansae, M. lanceolata* and *M. perlarius* was determined by means of TLC analysis. Callus material did not contain sufficient amounts of saponin for TLC detection. In contrast, the saponin content of *in vitro* shoots was similar to that of leaves of greenhouse grown plants. Comparison of saponin content of hairy roots with roots from *in vitro* and greenhouse plants showed only small differences. To investigate the impact of plant hormones on the production of saponins, leaves and shoots were treated and subsequently analysed. 2.4-D, ABA and GA3 did not influence the saponin production within in a reproducible fashion. A second series of experiments were done to analyse het effect of potential elicitors. In this case, hairy roots were used because these produce relatively low amounts of saponin. Also here no significant effect on saponin production was observed. Taken together, the results imply that saponins in *Maesa* species are constitutively produced and hence belong to the class of phytoanticipins.

4.2 Introduction

Plant cell and tissue cultures offer a system for production of secondary products independent from fluctuations in supply of the raw material that might have been created by changes in the climate, agriculture and political activities of the source country. However, only very few species are currently commercially produced through cell or tissue cultures. The reason for this is the difficulty to satisfy the commercial and biological criteria imposed on the product i.e. a high value, strong commercial demand, high yield in culture and maintenance of a high yield in large-scale culture. The last two criteria are common problems in tissue culture, therefore several strategies for improving secondary metabolite production have been developed; one of them comprises elicitation (Georgiev et al. 2007; Jeong and Park 2006).

For *Maesa argentea, M. balansae, M. lanceolata* and *M. perlarius* different *in vitro* cultures and propagation methods were successfully established (Chapter 3). However, the saponin production in these *in vitro* cultures was not yet studied in detail. The secondary metabolite production in *in vitro* cultures of plants, cells or tissues can increase or decrease depending on the nutrient composition of the culture medium (Collin 2001). Especially the type and concentration of phytohormone is an important

factor influencing their potential for secondary product synthesis. For example, the synthetic auxin 2,4-D can stimulate cell division and cell expansion but can as well cause a strong suppression of the secondary metabolism (Morris et al. 1985). In contrast, there are reports where auxins, cytokinins or abscisic acid are applied as 'elicitors' for secondary product production (Kim et al. 2007; Liu et al. 2007; Luo et al. 2001). Also the plant defence related phytohormones methyl jasmonate and salicylic acid are often used to enhance secondary metabolite biosynthesis. They are considered as wide spectrum elicitors due to their role in signal transduction pathways (Creelman and Mullet 1997; Raskin 1992). In addition to these hormones, there are many other substances that can influence the secondary product formation in plant systems; for example $CaCl_2$ (Yue and Zhong 2005), fungal elicitors (Pereira et al. 2007; Satdive et al. 2007; Sim et al. 1994; Xu et al. 2005), yeast extract (Lee et al. 1998; Pitta-Alvarez et al. 2000; Suzuki et al. 2005), chitosan (Palazon et al. 2003; Putalun et al. 2007) and heavy metals (Jeong and Park 2006).

In this chapter, the saponin production in different *in vitro* cultures was analyzed in more detail and compared with saponin production in greenhouse grown plants using TLC. In addition we investigated the influence of phytohormones on the saponin production in *in vitro* shoot cultures of all four *Maesa* species. For *Maesa lanceolata* hairy roots we set up a screen, using HPLC-MS analysis, to investigate putative elicitors of saponin biosynthesis.

4.3 Results

4.3.1 Saponin production in *Maesa in vitro* cultures

Saponin content was determined for *in vitro* cultures of shoots, hairy roots and callus of *M. argentea, M. balansae, M. lanceolata* and *M. perlarius* using thin layer chromatography (TLC) (Fig 4-1). For all species, the *in vitro* shoot cultures were started from aseptic seeds and were subcultured every 4 to 6 weeks during 1 year. The callus material was for the four species induced on *in vitro* leaves by placing them on 2,4-D containing culture medium. The calli were subcultured every 2 weeks for 1 year. Hairy roots were induced on leaves of *M. lanceolata* through *Agrobacterium rhizogenes* infection. Hairy root cultures were about 2 years old and were subcultured every month. More information on the *in vitro* shoot cultures and calli can be found in Chapter 3 and hairy root induction will be described in more detail in Chapter 5.

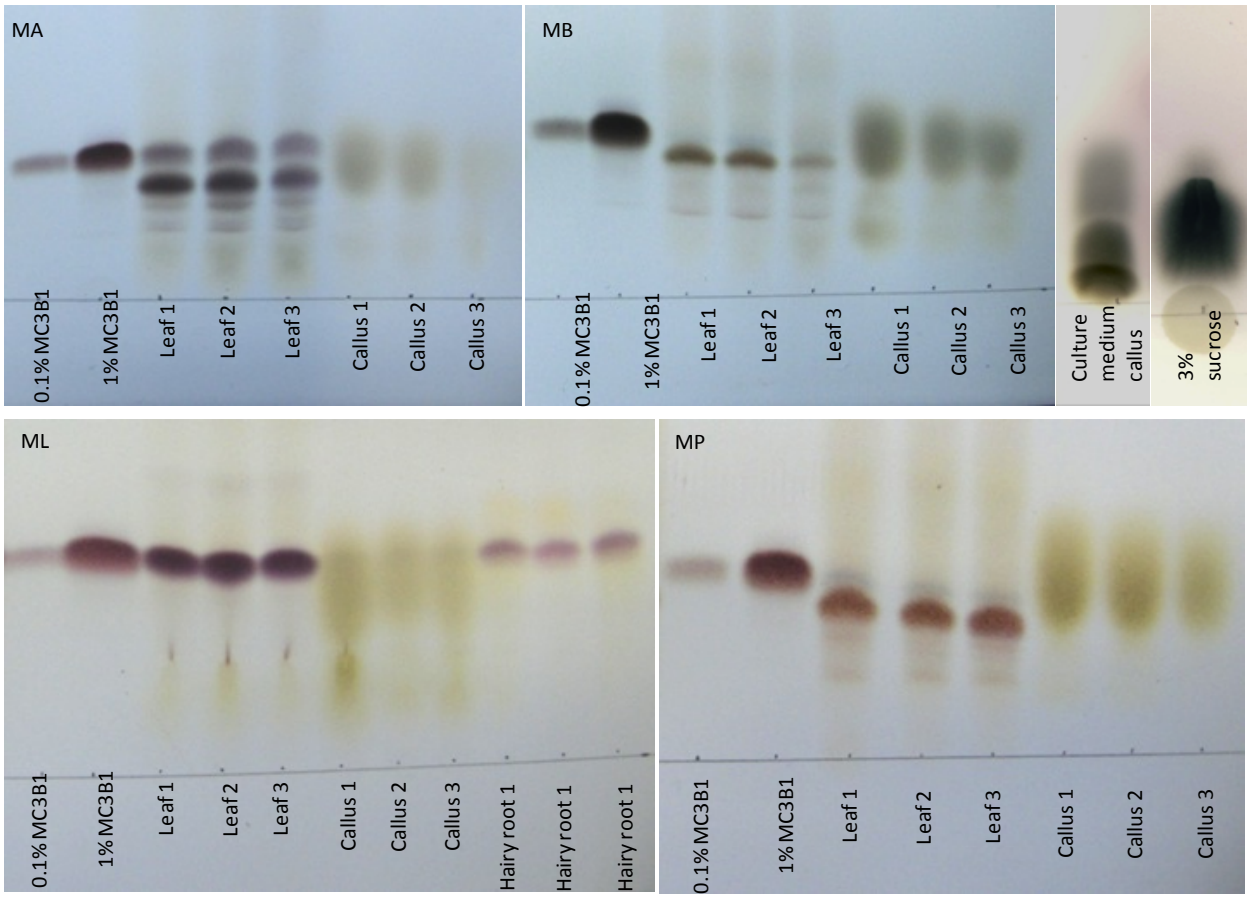


Fig 4-1 TLC of saponins in different types of *in vitro* cultures (*in vitro* shoots and callus) for *M. argentea* (MA), *M. balansae* (MB), *M. lanceolata* (ML) and *M. perlarius* (MP). Additionally, for *M. lanceolata* the saponin content in hairy roots was investigated. Two TLC lanes were included for the culture medium of the callus and 3% of sucrose to explain the background staining with the callus material. A HPLC purified maesasaponin mix (MC3B1) was used as reference sample. Different repeats represent different plants, calli or hairy root lines.

M. argentea in vitro shoots generated two major bands, one higher band with a retention factor (R_f) of 0.17 and one lower band with a R_f of 0.14. Two minor bands with a R_f of 0.12 and 0.07 were also detected. *M. balansae* shoots had one major band with a R_f of 0.16 and two very small lower bands with a R_f of 0.11 and 0.08. In addition, a weak blue band with a R_f of 0.17 was detected. For *M. lanceolata* only one band was detected (R_f = 0.17), which was the same in shoots as in hairy roots. In hairy roots the signal was much weaker suggesting a lower saponin content in hairy roots compared to shoots. *M. perlarius* shoots showed one prominent band with a R_f of 0.16 and two smaller ones with the same colour and retention factors of 0.14 and 0.16. Similar to *M. balansae* one weak blue band with a R_f of 0.18 was present. In conclusion, for *in vitro* shoots and hairy roots of *Maesa* species, we were able to detect two major bands: one with R_f 0.16-0.17 and one with R_f 0.14. It is likely that these bands are saponins because both the colour and the retention factor were similar to those of the reference sample.

The reference sample was a HPLC purified mixture of saponins from *M. lanceolata*. In addition, it was remarkable that the TLC patterns for *M. balansae* and *M. perlarius* were very similar.

In callus extracts a large brown spot was observed after TLC development (Fig 4-1). Because callus is in direct contact with the sucrose during culturing and sugars can react with p-anisaldehyde, the growth medium was analyzed with TLC. In addition, all the components in the culture medium were separately tested on TLC (data not shown). The medium, as well as the pure sucrose solution, produced a signal similar to what was detected in callus extracts. It is therefore more likely that the signal detected in callus extracts does not correspond to the presence of saponins but to sucrose and sugars. Similar signals were detected for callus samples from all four species.

In a subsequent experiment, saponin content of *in vitro* shoots was compared with saponin content of greenhouse grown plants (Fig 4-2). This experiment was performed in order to investigate if the *in vitro* culturing had an impact on the saponin content of the plants. We compared the saponin content of *in vitro* plants with greenhouse plants of three different ages (1 month, 9 months and 24 months).

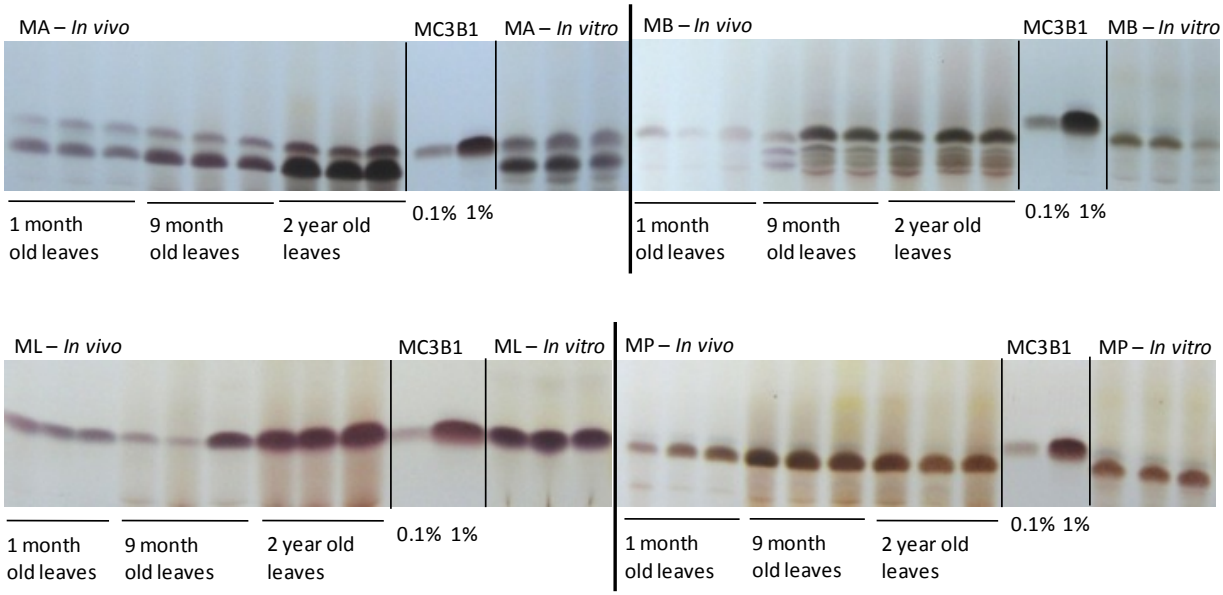


Figure 4-2 TLCs of saponins produced by greenhouse grown plants with different ages (1 month, 9 months and 2 years old plants) and saponins produced by *in vitro* plantlets for *M. argentea* (MA), *M. balansae* (MB), *M. lanceolata* (ML) and *M. perlarius* (MP). A HPLC purified maesasaponin mix (MC3B1) was used as reference sample. Different repeats represent separate plants.

Saponin production in greenhouse plants increased with increasing age of the plants for all species, these observations were already discussed in Chapter 2. *M. argentea in vitro* plants had a saponin content which was comparable with plants that were in the greenhouse for 9 months. For *M. balansae in vitro* plants, the saponin content was between that of 1 and 9 months old greenhouse plants. Finally, *M. lanceolata* and *M. perlarius in vitro* plantlets produced saponins comparable to 2 year old greenhouse grown plants. For all species, the retention factors of *in vitro* plants and greenhouse grown plants were the same. So from this experiment we conclude that *in vitro* cultivated *Maesa* species have a similar capacity to produce saponins as greenhouse grown plants.

In the next series of experiments we compared the saponin content of *Maesa lanceolata* hairy roots with the saponin content in roots of greenhouse grown and *in vitro* plants (Fig 4-3). Hairy roots are often used as a tool for stable production of secondary metabolites and it is often claimed that phytochemical production in hairy roots is quantitative and qualitative similar to that in normal roots (Giri and Narasu 2000; Hu and Du 2006).

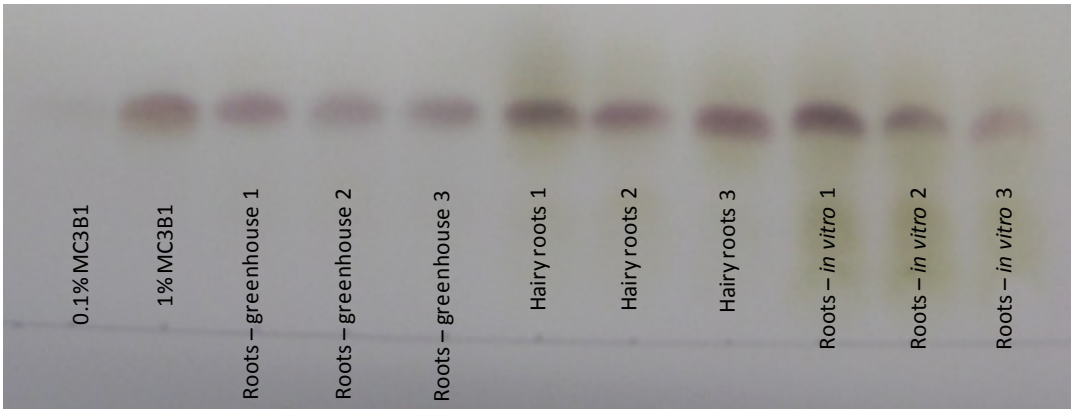


Figure 4-3 TLCs of *Maesa lanceolata* saponin extracts from the roots of greenhouse grown plants, the roots of *in vitro* plants and hairy roots. A HPLC purified maesasaponin mix (MC3B1) was used as reference sample. Different repeats represent independently isolated plants and culture samples.

Saponin content in the different types of *Maesa lanceolata* root tissues was similar. It seemed however that the concentration was somewhat higher in hairy roots and roots from *in vitro* plantlets than in the roots of greenhouse grown plants. The R_f of all spots was 0.17, which suggested that there were no qualitative changes.

4.3.2 Influence of phytohormones on the saponin production in *Maesa in vitro* shoot cultures

Phytohormones have a strong impact on the physiology and development of plants and they are very often used in tissue culture techniques. Therefore it would be interesting to see if these hormones also can influence saponin biosynthesis in *Maesa* species. In addition, there are reports that describe the upregulation of secondary metabolites upon treatment with phytohormones (Kim et al. 2007; Liu et al. 2007; Luo et al. 2001; Mahady et al. 1998; Zhao et al. 2005). *In vitro* shoots of all four *Maesa* species were submerged in an aseptic hormone solution for 30 seconds and subsequently placed on fresh medium. Gibberellic acid (GA3), 2,4-dichlorophenoxyacetic acid (2,4-D) and abscisic acid (ABA) were tested in a concentration of 10mM and samples were harvested 48 hours after treatment (Fig 4-4). For saponin analysis saponins were extracted from whole plants.

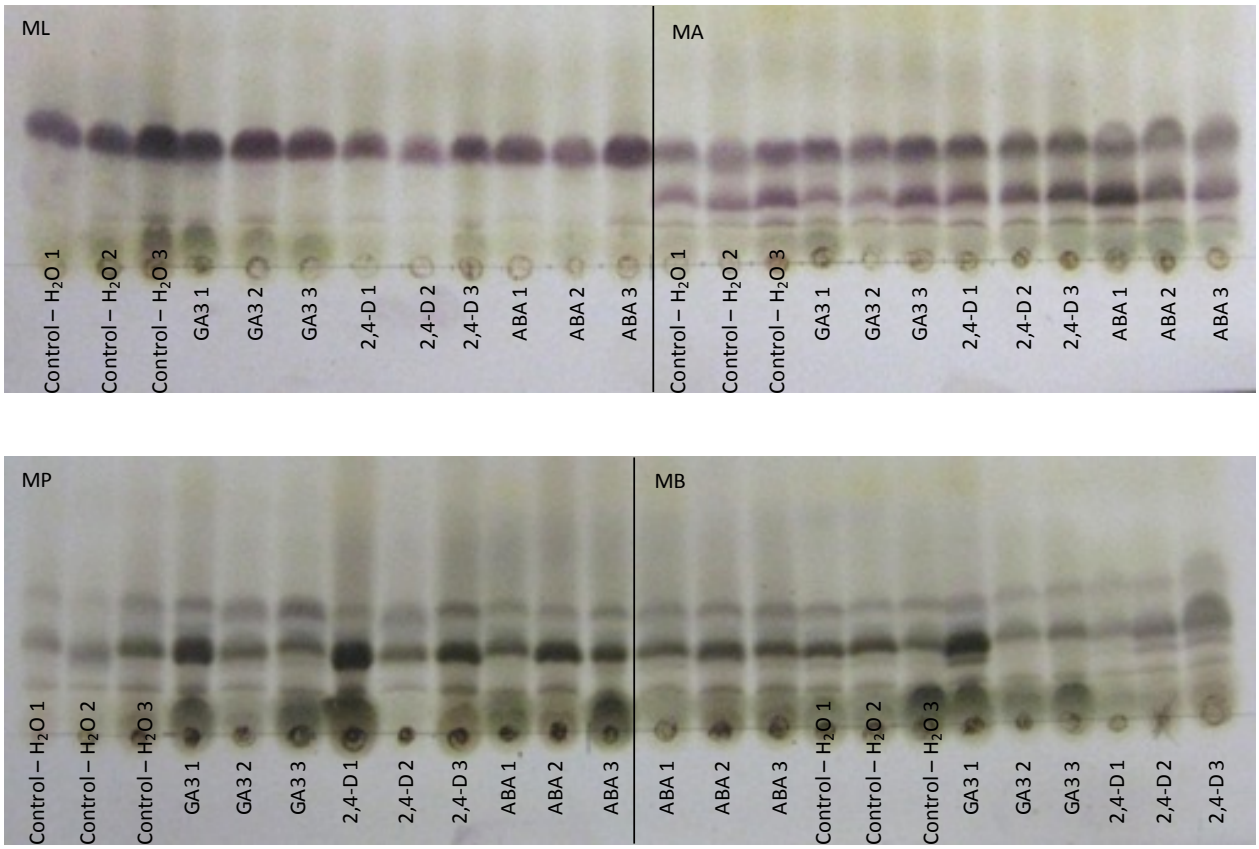


Fig 4-4 TLC of *in vitro* plantlets of *M. argentea* (MA), *M. balansae* (MB), *M. lanceolata* (ML) and *M. perlarius* (MP) upon treatment with different hormones; 0.01mM gibberellic acid (GA3), 0.01mM 2,4-dichlorofenoxyacetic acid (2,4-D) and 0.01mM abscisic acid (ABA). Controls were treated with water and all samples were harvested 48 hours after elicitor treatment. Different repeats represent separate plants.

In all control extracts we noticed some differences in saponin concentration. The origin of this variability is not clear. The differences between the controls and the treated samples were therefore considered as variability due to the extraction and/or TLC procedure. Because no major changes were observed for the different treatments, we assume that a short hormone treatment does not influence saponin accumulation. The additional bands that were observed for *M. balansae* and *M. perlarius* compared to Figure 4-1 and 4-2 were due to the root material that was included in these extracts (analysis of saponin content in different organs of all species is described in Chapter 2).

Jasmonic acid (JA) and methyl jasmonate (MeJA) are cyclopentanone-based compounds that are widely distributed in the plant kingdom. Exogenous applied JA or MeJA has an influence on e.g. vegetative development, fruit development and pollen viability, and jasmonates are therefore regarded as a new class of phytohormones. JA and MeJA are also involved in plant defense responses and, because of that, are widely used for enhancing secondary metabolites that are involved in protection of plants against pathogens, like saponins (Creelman and Mullet 1997). The effect of 0.1mM MeJA on *in vitro* shoots of all four *Maesa* species was investigated using TLC (Fig 4-5). Plantlets were dipped in a solution for 30 seconds and then placed on fresh medium. Additionally, we tested the effect of acetic acid, formic acid and citric acid (Fig 4-5). In a patent application it is described that acetic acid can enhance the secondary metabolite production in many different plant species (Raskin and Poulev 2002)(US 2002/0132021 A1). The mechanism of action of acetic acid is not known; therefore we included two other acids to be able to distinguish between a direct effect of acetic acid and a pH effect.

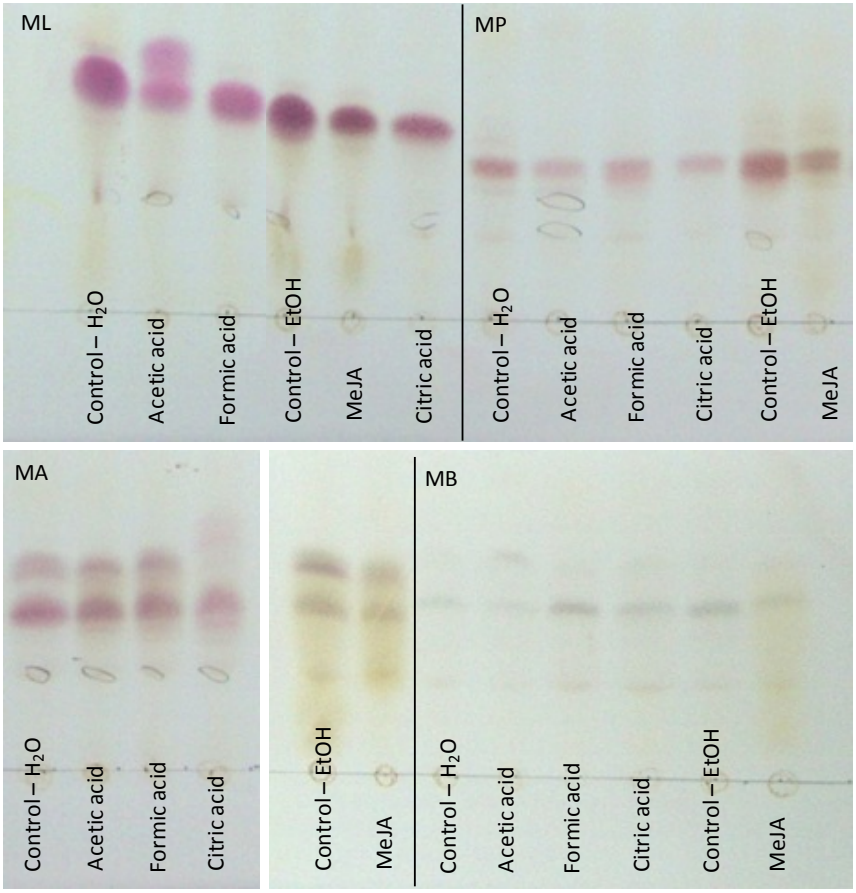


Fig 4-5 TLCs of *in vitro* plantlets of *M. argentea* (MA), *M. balansae* (MB), *M. lanceolata* (ML) and *M. perlarius* (MP) after elicitation with 10mM acetic acid, 10mM formic acid, 10mM citric acid and 0.1mM methyl jasmonate (MeJA). Control leaves were treated with H_2O and with ethanol (EtOH, solvent of methyl jasmonate). Samples were harvested 48 hours after elicitor treatment.

For both *M. argentea* and *M. perlarius* none of the treatments seemed to give a larger band on TLC compared to the controls. For *M. balansae* only very weak bands could be detected which were more strong for ethanol, formic acid and citric acid treatment. For *M. lanceolata* it appeared that there were no quantitative changes in saponin production. However, acetic acid induced a second band on the TLC. In a follow up experiment this additional band was no longer detected. *M. lanceolata* shoots were analyzed at different time points after acetic acid treatment (Fig 4-6).

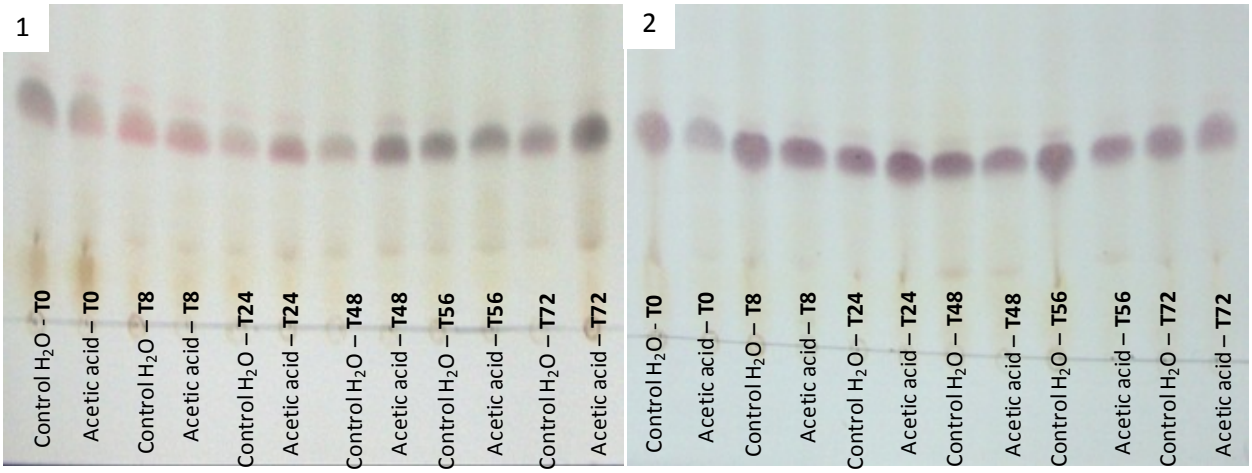


Fig 4-6 TLC of *in vitro* plantlets of *M. lanceolata* after elicitation with 10mM acetic acid. Samples were harvested at different time points, namely immediately (T0), 8 hours (T8), 24 hours (T24), 48 hours (T48), 56 hours (T56) and 72 hours (T72) after treatment with acetic acid. The two pictures are two biological repeats of the same experiments.

In contrast to the pilot experiment, none of the time points produced a second band on the TLC. In conclusion, these two experiments indicated that MeJA and the tested acids have no or only a limited influence on saponin production.

SA too is often used for the elicitation of secondary products, however, SA seems to act more selective then MeJA (Zhao et al. 2005). *Maesa in vitro* plants were submerged in an aseptic SA solution for 30 seconds, samples were harvested 48 hours after treatment and saponin production was examined with TLC (Fig 4-7).

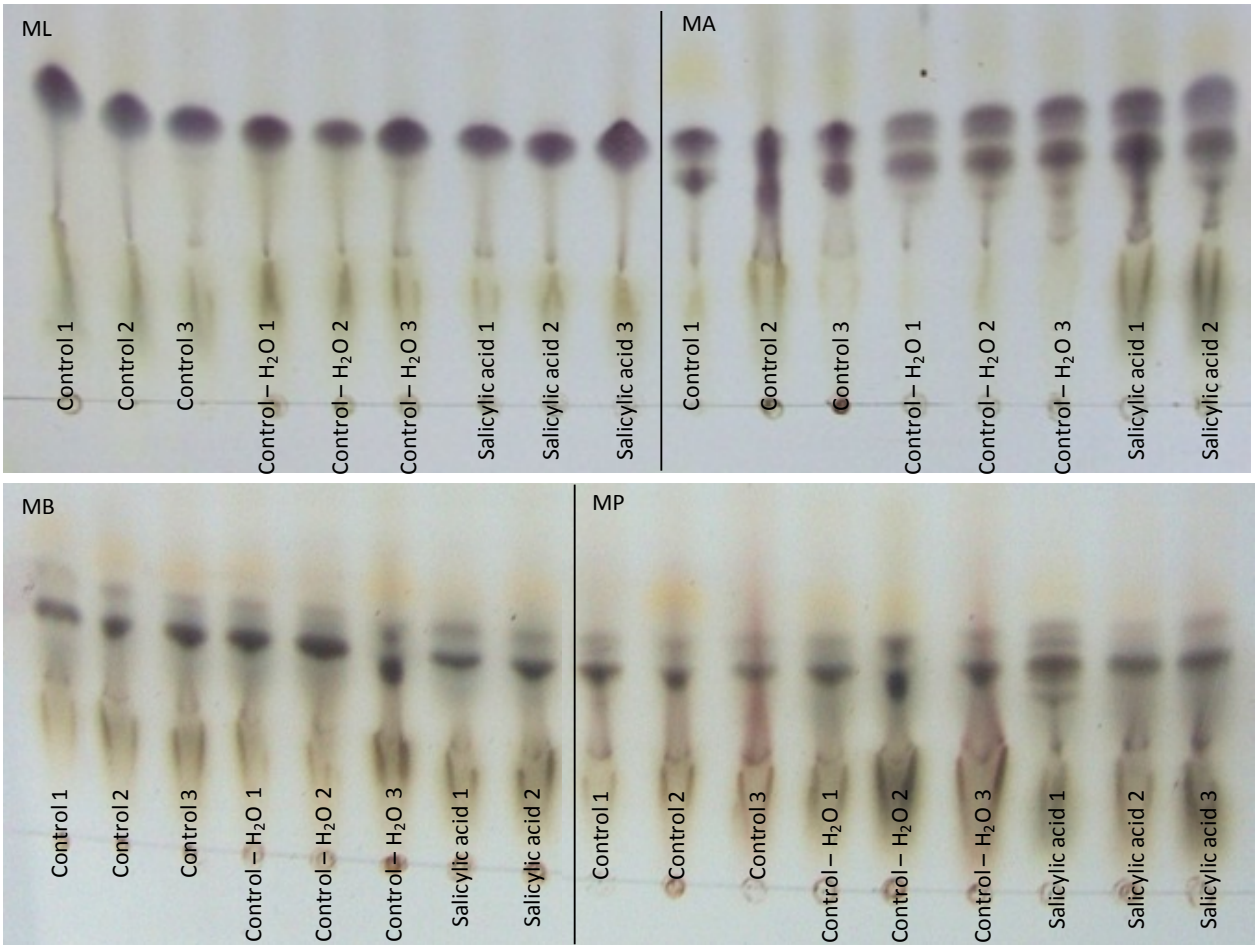


Fig 4-7 TLC of *in vitro* plantlets of *Maesa argentea* (MA), *M. balansae* (MB), *M. lanceolata* (ML) and *M. perlarius* (MP) treated with 0.01mM salicylic acid for 48 hours. As a control, non-treated leaves and leaves treated with water were used. Different repeats represent different plants.

After treatment with salicylic acid there were no changes in saponin content for none of the species. The results are in agreement with a stable accumulation of saponins in *Maesa* plants, not altered by externally applied phytohormones.

4.3.3 A preliminary screen of putative elicitors of saponin biosynthesis in *Maesa lanceolata* hairy roots

Hairy roots are often used for the production of saponins because they offer many advantages over other types of *in vitro* cultures. Hairy roots grow fast without need of exogenous hormones in the culture medium and they are easy to scale up in larger tissue culture containers. Moreover, hairy roots are capable of producing phytochemicals that are also produced in normal roots (Giri and Narasu 2000; Hu and Du 2006). *Maesa lanceolata* hairy roots contained saponins in a concentration comparable to roots of greenhouse grown and *in vitro* plants but in a concentration lower than leaves of greenhouse grown and *in vitro* plants. Thus, the saponin content could potentially increase to the same levels as had been found for shoots. We tried to enhance the saponin production in *Maesa lanceolata* hairy roots using many types of elicitors. Different treatments included **controlled infection** with fungal and yeast components, treatment with **acids**, treatment with **hormones** (mainly methyl jasmonate but also salicylic acid, auxins, cytokinins and abscisic acid), treatments with **chemicals** and **permeabilization**. For elicitation, hairy roots were transferred to liquid medium and elicitors were added one week later. More details on duration of treatment and concentration of elicitors can be found in Addendum I. Total saponin extracts of treated hairy roots were analyzed directly using HPLC-MS. HPLC-MS measurements were performed by the Lab of Pharmacognosy and Pharmaceutical analysis (University of Antwerp). Results are included in Addendum I.

The goal of the experiment was to perform a screen of many different kinds of putative elicitors that would enhance saponin production in *M. lanceolata* hairy roots. Because of financial and time constraints experiments were performed without biological repeats. The total saponin content of the hairy roots and the culture medium was determined.

HPLC-MS analysis revealed small variations in saponin content of treated hairy roots and the corresponding culture medium compared to control hairy roots. The highest increase in saponin production in the hair roots (2 x) was obtained by using 1.0 mM $CdCl_2$. In the culture medium the maximum increase was also 2 fold, after treatment with 1.0 mM MeJA. Because none of the tested compounds gave a strong induction of saponin production or secretion, we performed no further analysis.

4.4 Discussion

The importance of natural products in drug development and pharmaceutical industry, with about 25% of all the drugs prescribed worldwide being of plant origin, is well established (Rates 2001). However, the potential use of plants as a source of new drugs is still poorly explored. Only a small number of plants have been investigated phytochemically and even a smaller percentage has been studied in terms of pharmacological properties. *Maesa* species are widely used in traditional medicine and recently it was found that they produce pharmaceutically interesting saponins (Foubert et al. 2008; Leonard et al. 2003; Sindambiwe et al. 1996). However, classical cultivation and breeding of *Maesa* spp. is hindered because of practical problems, thus, we established *in vitro* cultures for the propagation and conservation of these plant species. It is described that *in vitro* conditions can influence secondary metabolite production; therefore, a thorough investigation of the saponin content in the established *in vitro* cultures is necessary before these cultures can be considered as production vehicles for triterpene saponins (Morris et al. 1985).

Callus material of *Maesa argentea, M. balansae, M. lanceolata* and *M. perlarius* did not contain sufficient amounts of saponin for detection on TLC. A generally observed phenomenon is that production of secondary metabolites is low in undifferentiated tissue (Bourgaud et al. 2001; Collin 2001; Wink 1989). Although there are some publications in which cell cultures are successfully used for the production of secondary metabolites (Pereira et al. 2007; Suzuki et al. 2002; Wang et al. 2005; Yue and Zhong 2005), there are also reports that describe a decrease in secondary metabolite production in de-differentiated tissue (Baiza et al. 1998; Pinol et al. 1999; Yamamoto and Kamura 1997). *Maesa* calli were grown on 2,4-D containing medium. Phytohormones in general, and 2,4-D in particular, can have a strong negative effect on secondary metabolite production in plant cell cultures. One of the major factors contributing to the low yields of secondary products in cell cultures reported before the seventies, was the general use of 2,4-D in the culture media (Morris et al. 1985).

In contrast to callus material, shoots of *in vitro* plantlets produced amounts of saponins similar to *ex vitro* grown plants. For leaves *M. lanceolata* greenhouse grown plants it is reported that they contain 4.9% saponins (Theunis et al. 2007). Comparing the saponin extracts of plants grown in our greenhouse on TLC with a HPLC purified maesasaponin mixture (MC3B1), we could conclude that our plants probably did not contain similar high amounts of saponins. We did see a large difference in saponin content of plants with different ages. It is clear that there is a large variation in saponin production depending on physiological

status of the plants. In addition, saponin production may be dependent on environmental conditions (Hostettmann and Marston 1995). Both these factors could be a source for variability in production that was observed.

Phytohormones play an important role in plant physiology, growth and development (Gray 2004) and hence potentially influence secondary metabolite production. *Maesa in vitro* shoots were treated with three different types of hormones; gibberellic acid (GA3), abscisic acid (ABA) and the auxin 2,4-D. All three phytohormones induced saponin production in single plants; however, the effect was not recurrent in all samples and was therefore not considered significant. A rather small effect of treatment of plant hormones on saponin production was also observed for Ginseng adventitious roots. Treatment with 0.025mM IBA increased saponin content 1.6 times (Kim et al. 2007). In *Panax ginseng* and *P. japonicus* cell cultures, the content of saponins varied depending on the hormones in the culture medium. The results suggested that saponin accumulation depended stronger on the used auxin than cytokinin (Smirnova et al. 2010). On the other hand, for anthocyanin accumulation in callus cultures of *Oxalis linearis* it was found that cytokinins had an enhancing effect while auxins had a repressing effect (Meyer and Vanstaden 1995). Cytokinins also seemed to have a stimulatory effect on the production of secondary products in *Hypericum sampsonii* and *H. perforatum* plantlets (Liu et al. 2007). For *Maesa* species we observed that long term treatment with auxin and subsequent callus formation reduced saponin content. In further experiments it would be interesting to investigate how the saponin content is reduced, either via the dilution as a consequence of cell division or by an active degradation.

Methyl jasmonate (MeJA) is besides a phytohormone also a very potent elicitor in plant cultures. Treatment of *in vitro* plants of all four *Maesa* species with MeJA did not cause an increase in saponin concentration, suggesting no or only a weak effect of MeJA on the biosynthesis. These very low changes in saponin production upon MeJA treatment in *Maesa* are in contrast with MeJA effects on secondary metabolite production. For MeJA elicitation often very high increases of saponin production are noticed in a wide variety of plants and culture types; a 10 fold increase of saponin production is observed in *Medicago truncatula* cell cultures (Suzuki et al. 2002), 7 times in *Centella asiatica* hairy roots (Kim et al. 2007), 6 times in *Azadirachta indica* hairy root cultures (Satdive et al. 2007), 4 to 6 times in *Centella asiatica* roots of whole plants (Mangas et al. 2006), 4 times in *Panax ginseng* hairy roots and adventitious roots (Palazon et al. 2003), 4 times in *Glycyrrhiza glabra in vitro* plantlets (Shabani et al. 2009) and 3 times in *Panax notoginseng* cell cultures (Hu and Zhong 2008).

The phytohormone salicylic acid (SA) is a well known inducer of plant systematic acquired resistance in plant-pathogen interactions. Therefore, SA is also often used to enhance secondary metabolite production in plant cultures. Treatment with SA increased saponin content 3 times in *Panax ginseng* adventitious roots (Ali et al. 2006) and 4.5 times in *in vitro* plant cultures of *Glycyrrhiza glabra* (Shabani et al. 2009). Even a 9 fold induction of secondary products was reported for *Azadirachta indica* hairy root cultures (Satdive et al. 2007). *Maesa argentea* and *M. balansae in vitro* plants had a thicker band on TLC, however, this was only observed in one out of the three repeats. For *M. lanceolata* and *M. perlarius* no effect of SA was observed. Unlike MeJA, SA is not a universal elicitor of secondary and defensive metabolites (Zhao et al. 2005), which is a possible explanation for the very small effect of SA on saponin content in *Maesa* species.

Finally, also the effect of acetic acid on saponin production in *Maesa in vitro* shoots was tested. Preliminary tests with *M. lanceolata* hairy roots showed that addition of acetic acid to the culture medium increased foaming of the culture medium, which could be a sign of higher saponin production and secretion (data not shown). The idea to test acetic acid was based on a patent application that describes acetic acid as an effective elicitor for a large range of plant species and secondary metabolites (Raskin and Poulev 2002)(US 2002/0132021 A1). In addition to acetic acid, other acids were tested for elicitation of *in vitro* plants to differentiate between a specific effect of acetic acid and a non-specific effect of a lower pH of the culture medium. However, for *Maesa in vitro* plantlets acetic acid had no or only a weak effect on saponin accumulation.

Maesa lanceolata wild type hairy roots contained saponins in a concentration of approximately 2% of the dry weight. TLC analysis revealed that the saponin content of hairy roots was more or less the same as in roots of *in vitro* and greenhouse grown plants. Comparison of hairy roots with shoots of *in vitro* and greenhouse plants, however, showed that hairy roots contained a lower concentration of saponins than shoots. In spite of this lower saponin content, we believe that hairy roots are a very good tool for the production of secondary metabolites; they grow fast in hormone-free medium and they can be scaled up in bioreactors (Kim et al. 2002). In addition, hairy roots are differentiated tissues so they are generally stable on a genetic and biochemical level (Giri and Narasu 2000).

Maesa lanceolata hairy roots were subjected to a multitude of putative elicitors in different concentrations. However, it seemed that none of the substances induced saponin production. Treatment of *M. lanceolata* hairy roots with 1 mM $CdCl_2$ increased saponin content in the hairy roots 2 times and in

the culture medium 1.4 times. This was the strongest induction observed during hairy root elicitation experiments. This result is in contrast with the effects described in *Centella asiatica in vitro* plantlets, where saponin content decreased upon treatment with 5 mM $CdCl_2$ (Kim et al. 2004). A positive effect of $CdCl_2$ was described for tropane alkaloid production in *Atropa belladonna* hairy roots, which were also treated with 5 mM $CdCl_2$. In this last study it was noticed that cadmium also showed a permeabilization effect; addition of $CdCl_2$ led to lysis and a serious decrease in tissue viability in transformed *Atropa* roots (Lee et al. 1998). This property of $CdCl_2$ could be the reason why we also see increase in saponin content in the culture medium of the treated hairy roots.

In this chapter we give further evidence that *Maesa* saponins are produced in a stable fashion irrespective of the environmental conditions. *In vitro Maesa* plantlets and *Maesa lanceolata* hairy roots were subjected to various elicitors but none of the treatments increased production or accumulation of saponins. We also demonstrate that saponin production in *Maesa in vitro* cultures is comparable to the production in *ex vitro* grown plants. Thus, *in vitro* plantlets offer a good system to study the production of saponins. In addition, treatment of hairy roots with different kinds of elicitors did not have a strong influence saponin production, which makes hairy roots a stable and robust system for further silencing and overexpression studies. Furthermore, in Chapter 2 we showed that saponin content was higher in more mature leaves and older plants. It is generally accepted that during leaf maturation two processes occur that are both hormonally regulated: cell division and cell expansion, respectively regulated through auxin and gibberellic acid. It can be assumed that auxin and/or gibberellic acid are also directly responsible for the increase in saponin production. However, a short contact of *in vitro Maesa* leaves with auxin or gibberellic acid did not significantly change saponin production. Consequently, the increased saponin production in mature *Maesa* leaves cannot be a direct effect of the higher auxin and/or gibberellic acid levels in these leaves.

4.5 Materials and methods

4.5.1 Plant material

M. argentea, M. balansae, M. lanceolata and *M. perlarius* were cultured *in vitro* as described in the Materials and methods section in Chapter 2.
Induction and culture of *M. lanceolata* hairy roots is described in detail in the Materials and methods section of Chapter 5.

4.5.2 Saponin extraction

For TLC analysis, saponins were extracted as described in the Materials and methods section of Chapter 2.

4.5.3 Thin layer chromatography (TLC)

The protocol for TLC analysis of saponins is described in the Material and methods section of Chapter 2.

4.5.4 High performance liquid chromatography – mass spectrometry (HPLC-MS)

HPLC-MS measurements have been performed by the Lab of Pharmacognosy and Pharmaceutical analysis (Faculty of Pharmaceutical Sciences – University of Antwerp). The protocol for *Maesa* sample preparation and HPLC-MS is described in Theunis et al. (2007).

4.5.5 Saponin studies in *Maesa in vitro* plants

Saponin content in different Maesa in vitro cultures

In this experiment, we compared saponin content of *in vitro* shoots, calli and hairy roots of different *Maesa* species on TLC. Starting from the apex, the 1^{st} – 6^{th} leaves were harvested from *in vitro* plants without removing the plants from the medium. For each species, three different plants with the same age were used as biological repeats. 2 – 3 calli from the same plate were also combined for each saponin extraction. For each species, three different plates were used for three repeats. Hairy root material was only available for *M. lanceolata* and 4 – 6 weeks old control roots were used. The repeats represent three independent control hairy root lines.

Saponin content in Maesa in vivo leaves

For greenhouse grown plants three 1 month old, three 9 months old and three 1 year old were used. From these plants 2nd and 3rd leaves (starting from the apex) were combined and used for saponin extraction. Saponin contents were investigated using TLC.

Saponin content in Maesa lanceolata hairy roots and roots of in vitro and in vivo plants

For this experiment, saponins of hairy roots, roots of *in vitro* plants and roots of *in vivo* plants were compared on TLC. Only *M. lanceolata* was included in this experiment. 4 – 6 weeks old hairy root cultures and three independent control lines were used. 1 month old greenhouse grown plants were removed from the soil (a mixture of sand and peat soil, AVEVE) and rinsed gently with water. The roots were removed for saponin extraction. Different repeats represent different plants. *In vitro* plants were also removed from the culture medium and were rinsed with water. The roots of 3 – 4 plants from the same batch were combined for one experiment. Three batches of roots were analyzed.

Hormone and acid treatment of Maesa in vitro shoots

Plantlets were removed from the culture medium in the tissue culture containers and were submerged in the elicitor or control solution for 30 seconds. Afterwards, every plant was placed into a glass tube containing solid MS medium supplemented with MS vitamins, 3% (w/v) sucrose and 0.8% (w/v) agar (Lab M plant tissue culture agar MC29, Amersham). The cultures were placed in a growth room with 16/8 light/dark conditions at 26°C. Samples (1st – 6th leaves, starting from the apex) were harvested for saponin extraction 48 hours after treatment, except for acetic acid. 1st – 6th leaves of *M. lanceolata* plantlets treated with acetic acid were harvested immediately after treatment and after 8, 24, 48, 56 and 72 hours. Saponin content was investigated with TLC.

2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma), Abscisic acid (ABA) (Fluka), Gibberellic acid (GA3) (Duchefa Biochemie B.V.) and Salicylic acid (SA) (Duchefa Biochemie B.V.) were tested in a concentration of 0.01mM and were dissolved in H_2O. Methyl jasmonate (MeJA) (Sigma-Aldrich) was used in a concentration of 0.1mM and was dissolved in 100% ethanol. Finally, 10mM acetic acid (Sigma), formic acid (UCB) and citric acid (Sigma) dilutions were made in H_2O. Details on different elicitation experiments are also represented in Addendum I.

4.5.6 Elicitor screen with *Maesa lanceolata* hairy roots

For elicitation, *M. lanceolata* hairy roots (+/- 5g fresh weight of 4 – 6 weeks grown control cultures) were transferred from petridishes to Erlenmeyers. The Erlenmeyers were filled with 10ml of liquid Schenk & Hildebrandt (SH) medium (Schenk and Hildebrandt 1972) supplemented with SH vitamins, 5mM myo-inositol (Duchefa Biochemie B.V.) and 3% (w/v) sucrose (pH 5.8). Cultures were grown in the dark at 25°C on a rotary shaker (120 rpm). Elicitors were added one week after transfer to liquid medium. Details on different elicitation experiments are also summarized in Addendum I.

Elicitors:

- Chitosan (Sigma-Aldrich) was dissolved in 100% acetic acid and was tested in a concentration of 50 mg/l, 100 mg/l, 150 mg/l, 200 mg/l and 250 mg/l. Samples were harvested after 1 week.

- A suspension of fungal spores was started from a *Botrytis cinerea* culture that formed spores. The culture was brought in H_2O and the suspension was shaken until the water became brown and turbid. The suspension was then filtered through a nylon filter to get a pure solution of fungal spores. The spores were counted in a Fuchs Rosenthal haemocytometer and the number of spores in one ml is calculated using the formula (number of spores * 1000)/0.8. The fungal spores were added in a concentration of 5 000 spores/ml, 10 000 spores/ml, 50 000 spores/ml and 100 000 spores/ml. Samples were harvested 48 hours after treatment.

- For inoculation with a mycelium homogenate, *Botrytis cinerea* was inoculated in liquid PDB medium, Potato Dextrose Broth medium. After inoculation, the bottles were placed horizontally in a dark room at 25°C. 1 week later the mycelium, which grew on the surface of the medium, was removed and was homogenized with a mixer and filtered using a nylon filter. The optical density was then measured at 595nm with a Uvikon Spectrophotometer. Samples were harvested 48 hours after treatment.

- Yeast extract was dissolved in H_2O and was tested in 5 concentrations; 0.5 mg/l, 4.0 mg/l, 5.0 mg/l, 7.5 mg/l and 10.0 mg/l. Samples were harvested 48 hours after treatment.

- Acetic acid (Sigma) and citric acid (Sigma) dilutions were made in H_2O and applied in a concentration of 15 mM. Samples were harvested 1 week after addition of elicitors.

- Methyl jasmonate (MeJA) (Sigma-Aldrich) was dissolved in 100% ethanol and tested in the following concentrations: 0.1 mM, 0.5 mM, 1.0 mM, 10.0 mM and 15.0 mM. Samples were harvested 48 hours after treatment.

- 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma-Aldrich), Benzyladenine (BA) (Sigma-Aldrich) and Abscisic acid (ABA) (Fluka) were dissolved in H_2O and applied in a concentration of 0.1 mM. Samples were harvested 48 hours after treatment.

- Naphthylphtalamic acid (NPA) (Duchefa Biochemie B.V.) was dissolved in 100% DMSO and tested in a concentration of 0.01 mM. Samples were harvested 72 hours after treatment.

- Salicylic acid (SA) (Duchefa Biochemie B.V.) was dissolved in H_2O and tested in two concentrations: 0.1 mM and 0.2 mM. Samples were harvested 48 hours after treatment.

- Benzothiadiazole (BTH) (Syngenta) was dissolved in H_2O and was applied in a concentration of 0.1 mM, 1.0 mM, 10.0 mM and 50.0 mM. Samples were harvested 48 hours after treatment.

- We also tried to combine different hormones, namely 0.1 mM MeJA + 0.1 mM 2,4-D, 0.1 mM MeJA + 0.01 mM NPA, 0.1 mM MeJA + 0.1 mM ABA, 0.1 mM SA + 0.01 mM NPA and 0.2 mM SA + 0.01 mM NPA. Treatments were performed during 48 hours.

- $CdCl_2.H_2O$ (VWR International) was dissolved in H_2O and applied in a concentration of 0.1 mM, 1.0 mM, 2.0 mM and 5.0 mM. Samples were harvested 48 hours after treatment.

- $CaCl_2.2H_2O$ (VWR International) was dissolved in H_2O and applied in a concentration of 0.1 mM, 5.0 mM, 10.0 mM, 15 mM, 20 mM and 100 mM. Samples were harvested after 48 hours.

- Hydrogen peroxide (H_2O_2) is diluted in H_2O to a concentration of 0.1 mM, 0.5 mM, 1.0 mM and 5.0 mM. Samples were harvested 48 hours after treatment.

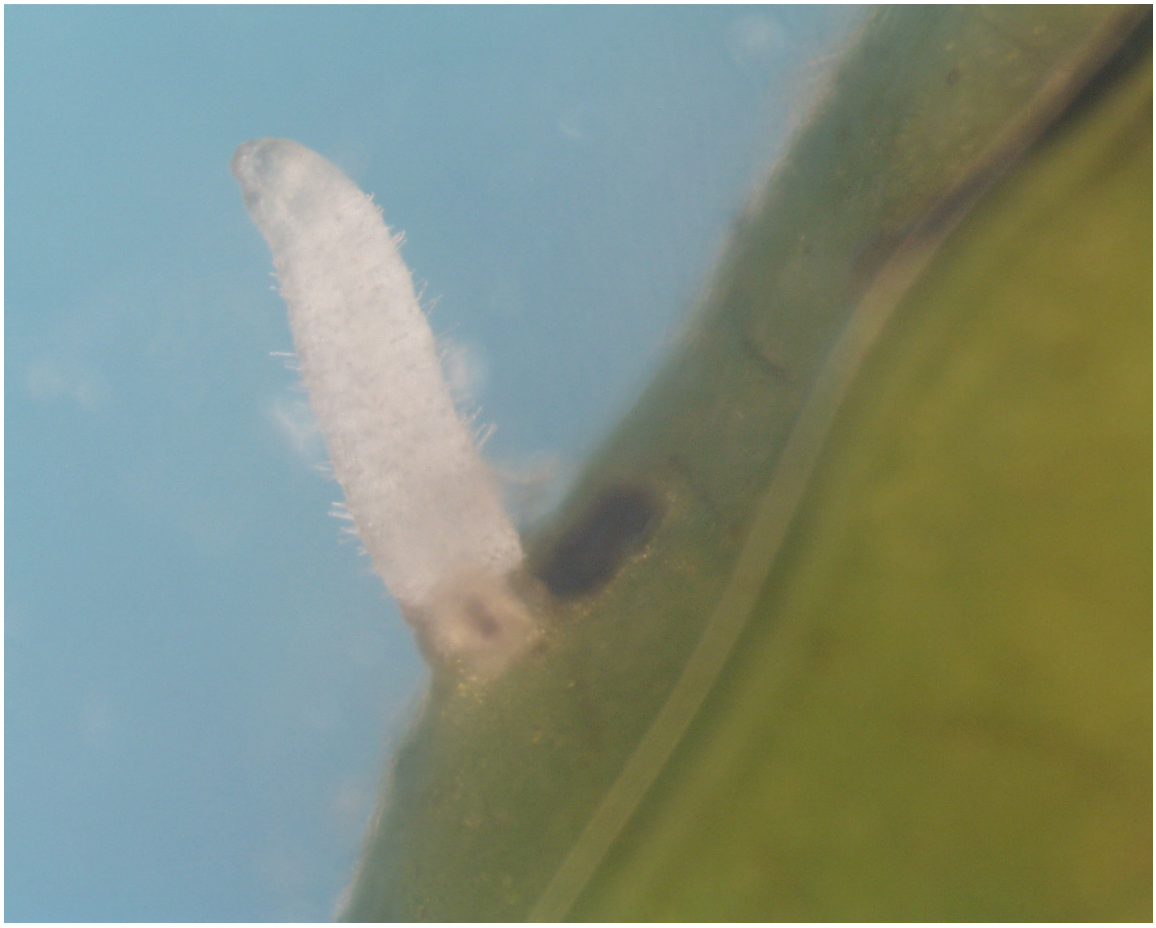
- Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich) was dissolved in 2.5% ammoniumhydroxide and applied in a concentration of 0.1 mM. Samples were harvested 48 hours after treatment.

- Tween-20 (Acros) and Triton X-100 (Aldrich) were tested in a concentration of 3% (v/v) and samples were harvested 48 hours after treatment.

- Dimethylsulfoxide (DMSO) was applied in three concentrations; 0.1% (v/v), 0.5% (v/v) and 1.0% (v/v) and samples were harvested 48 hours after treatment.

CHAPTER 5

A COMBINATORIAL BIOSYNTHESIS APPROACH IN MAESA LANCEOLATA HAIRY ROOTS



5.1 Abstract

The rational engineering of secondary metabolite pathways requires a profound knowledge of the biosynthetic pathway and a detailed understanding of the regulatory mechanisms. However, many secondary metabolites have multistep and complex biosynthetic pathways that are still largely uncharacterized. In addition, the plants producing pharmaceutically interesting secondary metabolites are often rare and tropical species for which there is no genome sequence knowledge and no standard protocols. We describe here, for the first time, a protocol for hairy root induction of *Maesa lanceolata*. The hairy roots were grown on solid medium, in liquid medium and in temporary immersion bioreactors for the upscaling of biomass production. Because the *Maesa lanceolata* genome is not yet determined, a semi-rational approach for changing the saponin production that did not require prior sequence knowledge, needed to be developed. Combiplan or COMbinatorial Biosynthesis in PLANts provided a platform for the development of these tools. In this chapter, *Maesa lanceolata* was transformed with RNAi constructs to silence endogenous genes and subsequently knock down certain metabolic conversions leading to particular natural substitutions or modifications of the saponin skeleton that might affect the activity of the maesasaponins. Unfortunately, this approach did not result in the modulation of saponin production and composition. In contrast to the ineffectiveness of RNAi, overexpression of a number of ORF's resulted in quite dramatic changes in saponin production. Several hypothesis are discussed that could explain these contrasting results.

5.2 Introduction

Hairy root is a plant disease caused by the gram-negative soil bacterium *Agrobacterium rhizogenes*. The bacterium carries a 'root inducing' (Ri) plasmid which contains 'transfer-DNA' (T-DNA) between two border regions or border repeats (Fig 5-1) (White and Nester 1980). When susceptible plants (mainly gymnosperms and dicotyledon species) are wounded, they produce phenolic substances, such as acetosyringone (AS). These substances are recognized by the *Agrobacterium* and make the bacterium chemotactic towards the AS and the wounded plant cells (Stachel et al. 1985). When the bacterium infects the plant, the T-DNA is transferred and integrated into the nuclear genome of the host plant (Zambryski 1988; Zambryski et al. 1989). The transformation process produces a by-product, hairy roots, which will form at or near the site of infection. In addition, opines (unusual amino acids) are produced and secreted into the soil. These serve as nutrients for the bacteria (Zambryski et al. 1989). Most importantly, *A. rhizogenes* can transfer T-DNA from binary vectors and enable the production of transgenic plants containing foreign genes carried on a second plasmid. This property has been used to produce transgenic plants (Tepfer 1984).

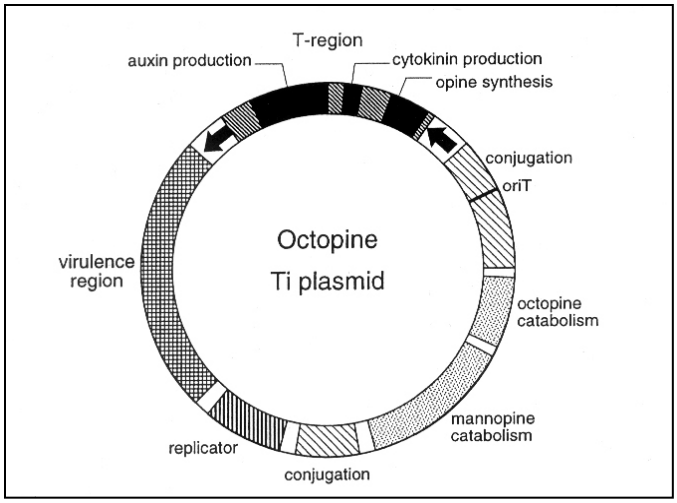


Fig 5-1 Genetic map of a tumor inducing (Ti) plasmid. A Ti plasmid is found in *Agrobacterium tumefaciens* and is the equivalent of the Ri plasmid in *Agrobacterium rhizogenes*. The arrows denote the border repeats. The T-region will be completely transferred to the plant genome. Within the T-region there are auxin and cytokinin producing genes, the ration of auxin to cytokinin will, amongst other mechanisms, determine the structures that are formed (callus, hairy roots or shoots) (White et al. 1985).

Hairy roots have relatively fast growth rate in hormone free media. Whereas other plant cell cultures have a strong tendency to be genetically and biochemically unstable and often synthesize very low levels of useful secondary metabolites, hairy roots are highly differentiated and can cause stable and high production of secondary metabolites. Recently, transformed roots are being used for the *in vitro* production of secondary metabolites (this has been extensively reviewed in (Giri and Narasu 2000) and (Georgiev et al. 2007)). Examples are the production of artemisinin in *Artemisia annua* hairy roots (Weathers et al. 2005), camptothecin in *Ophiorrhiza pumila* and *Camptotheca acuminata* hairy roots (Saito et al. 2001) and scopolamine and hysoscyamine in *Datura innoxia* hairy root cultures (Dechaux and Boitel-Conti 2005).

Combinatorial biosynthesis involves the basic concept of transferring genes encoding enzymes with a particular activity or substrate specificity from one species to another, not necessarily related, species with different potential substrates. In this way, opportunities for novel biosynthetic reactions are generated (Julsing et al. 2006; Khan et al. 2009). Combinatorial biosynthesis is not limited to the introduction of a single gene but can also include a series of genes with the aim to reconstruct a complete biosynthesis pathway that can impinge on an existing biosynthetic pathway in the host (Oksman-Caldentey and Inze 2004). This approach has already been successfully used for the metabolic engineering of microorganisms, especially *Streptomyces*, for the production of novel antibiotics, which proves the potential of combinatorial biosynthesis (Hranueli et al. 2005). However, such a strategy has not yet been implemented into a higher plant system because of the poor background knowledge of secondary metabolism and because of practical hurdles such as a slow life cycle compared to microorganisms. At the moment, combinatorial biosynthesis of plant secondary metabolites focuses on

the reconstruction of the basic pathways into microbial hosts. Recent achievements with the plant polyketide biosynthesis by microorganisms, especially in *Escherichia coli*, demonstrated the utility of combinatorial biosynthesis (Horinouchi 2009).

There are not yet reports about new structures of triterpene saponins based on combinatorial biosynthesis in plants. However, there are ample possibilities by which one could modify the basic structure of saponins in a given host species. A hypothetical example is the introduction of the dammarenediol synthase from *Panax ginseng* (*PgDDS*) into *Medicago truncatula*. In this way, a hybrid-type saponin could be created (Fig 5-2). Provided that combinatorial saponins are produced, the identification of saponin biosynthesis genes from different species will become more and more important in the future.

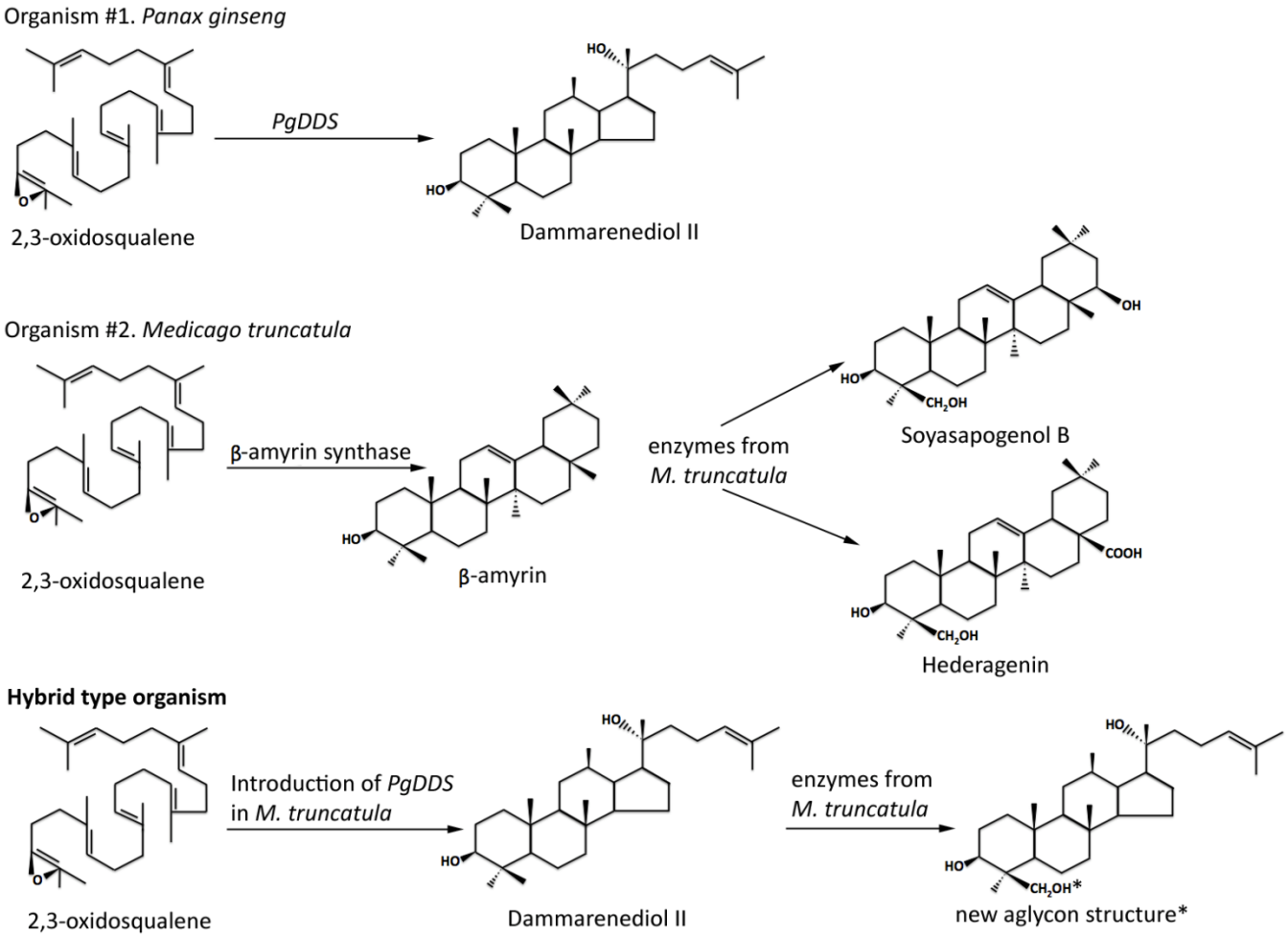


Fig 5-2 A hypothetical approach for combinatorial biosynthesis in higher plants. Introduction of the dammarenediol synthase gene from *Panax ginseng* (*PgDDS*) in *Medicago truncatula* could lead to the formation of dammarenediol II from the general precursor, oxidosqualene, in *M. truncatula*. This can then serve as a novel substrate for enzymes involved in the later steps of saponin biosynthesis in *M. truncatula*.

5.3 COMBIPLAN: COMbionatorial BIosynthesis in PLANts

The pharmaceutical industry is continuously searching for novel molecules with new or superior activities. There are various approaches and sources that can be used to fulfill this need for new structures. About two decades ago, natural product research efforts lost popularity with the development of a novel technique for high throughput chemical synthesis of small molecules, **combinatorial chemistry** (Muller-Kuhrt 2003). With this approach it was possible to synthesize large compound libraries which could serve as an indefinite source of products (Borman 2002). However, at the beginning of this millennium, natural product research regained interest, mainly because of a disappointing outcome of the high throughput combinatorial chemistry and the technical advances made in the field of natural product research (Muller-Kuhrt 2003; Oksman-Caldentey and Inze 2004). Much research has been focused on microbes; yet, plants are the source of the most complex individual mixtures of metabolites and therefore offer still an enormous opportunity for finding new biological active compounds. In addition, the emergence of plant genomics and a range of new transcriptomic, proteomic and metabolomic tools put a new perspective to natural product research. One novel approach that has originated the last decades is **combinatorial biosynthesis** (Oksman-Caldentey and Inze 2004). This technique offers the advantage that new compounds, with possibly novel and/or superior activities, can be synthesized that do not exist in nature (Muller-Kuhrt 2003).

The key objective of the Combiplan project was '*to establish a combinatorial biosynthesis platform in plants that will allow the semi-rational combinatorial engineering of the biosynthesis of existing and novel secondary metabolites in plant cell tissue cultures*'.

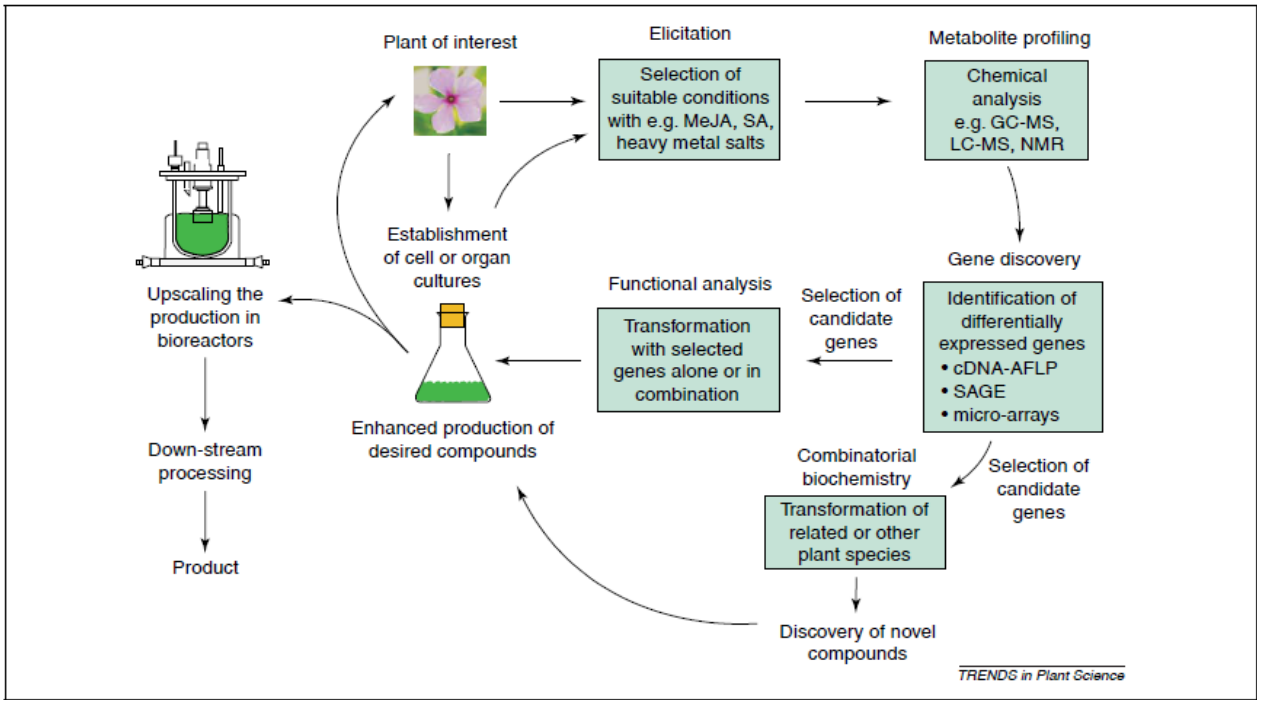


Fig 5-3 The Combiplan approach. Elicitors are added to cell cultures (or other tissue cultures) of plants of interest for a higher production of specific secondary metabolites, which can be detected through metabolite profiling. The rationale of the project is that an increase in secondary metabolites is preceded by an induction of biosynthesis genes involved in the production of these specific secondary metabolites. These genes will be differentially expressed upon elicitation and can be identified. Interesting genes will be cloned in an overexpression or RNAi cassette, to up- or downregulate the gene respectively. These cassettes can be transformed in the same plant species to enhance or decrease the saponin production, or in another plant species with related metabolites to make novel secondary metabolites. Figure adapted from Oksman-Caldentey and Inze (2004).

To obtain proof of concept, the designed strategy (Fig 5-3) was applied to the metabolite class of triterpene saponins. Six plant species were selected based on their saponin content: *Medicago truncatula, Panax ginseng, Maesa lanceolata, Aesculus hippocastanum, Bupleurum falcatum* and *Glycyrrhiza glabra*; with *M. truncatula* and *M. lanceolata* being the main plant species used. These species produce structurally related triterpene saponins, however, with varied structural features and different biological activities. First, novel and suitable host plant cell and tissue cultures were created and conditions for enhanced saponin production in plant tissues of the different species were established. The extraction and detection techniques for qualitative and quantitative metabolic profiling, based on chromatography and mass spectrometry, were optimized. In the meantime, high-throughput transformation and cryopreservation techniques were developed. Elicited plant material with increased saponin production was used for gene identification through cDNA-AFLP based transcript profiling and a collection of overexpression and RNAi cassettes was created for the generation of a combinatorial

biosynthesis library. Finally a screening procedure was set up to find novel metabolites and novel activities.

Maesa lanceolata shoot cultures were elicited with 500µM methyl jasmonate (MeJA) for the induction of saponins and samples were harvested at different time points (0, 0.5, 1, 2, 4, 8, 24 and 48 hours). A pilot cDNA-AFLP showed that the shoot cultures were MeJA responsive. Subsequently, a full genome-wide transcript profiling analysis was executed for MeJA treated and non-treated samples. The AFLP showed 13 558 tags, of which 733 showed differential expression between treated and non-treated samples. 312 were unique hits and after BLAST analysis 53 were identified as putative saponin biosynthesis and regulator genes. The group of genes consisted of oxidases, reductases, cytochrome P450s, glucosyltransferases, esterases, acyltransferases, transcription factors and some early pathway genes. 41 genes had a confirmed full length and 24 were cloned into an RNAi vector. This supporting work was executed in the Secondary Metabolites Group (Plants Systems Biology Department – VIB). More technical details are shown in the Materials and methods section. An overview of the genes that were eventually transformed in *M. lanceolata* is shown in Table 5-1.

In this chapter, the establishment of a protocol for hairy root induction on *M. lanceolata* hairy roots will be discussed, as well as the generation and analysis of *M. lanceolata* RNAi hairy root lines. In addition, we tested the growth of hairy roots in temporary immersion bioreactors and we investigated some RNAi lines that displayed a 'thick root' phenotype in more detail.

5.4 Results

5.4.1 An efficient protocol for *Maesa lanceolata* hairy root induction

Maesa lanceolata hairy roots were induced using *Agrobacterium rhizogenes* (strain LBA 9402/12) transformation on leaf discs. The *Agrobacterium* strain was transformed with the pK7WG2D plasmid, in which an eGFP-ER gene was inserted after a 35S promoter sequence (Materials and methods, Figure 5-9). The first step in hairy root induction was wounding the leaf material. Wounded leaves were dipped into the bacterial culture and afterwards placed on solid infection medium without antibiotics, which led to a co-culture of the bacteria and the leaves. After 3 days of co-cultivation with the *Agrobacteria,* the leaves were placed on selective cultivation medium with antibiotics to arrest bacterial growth.

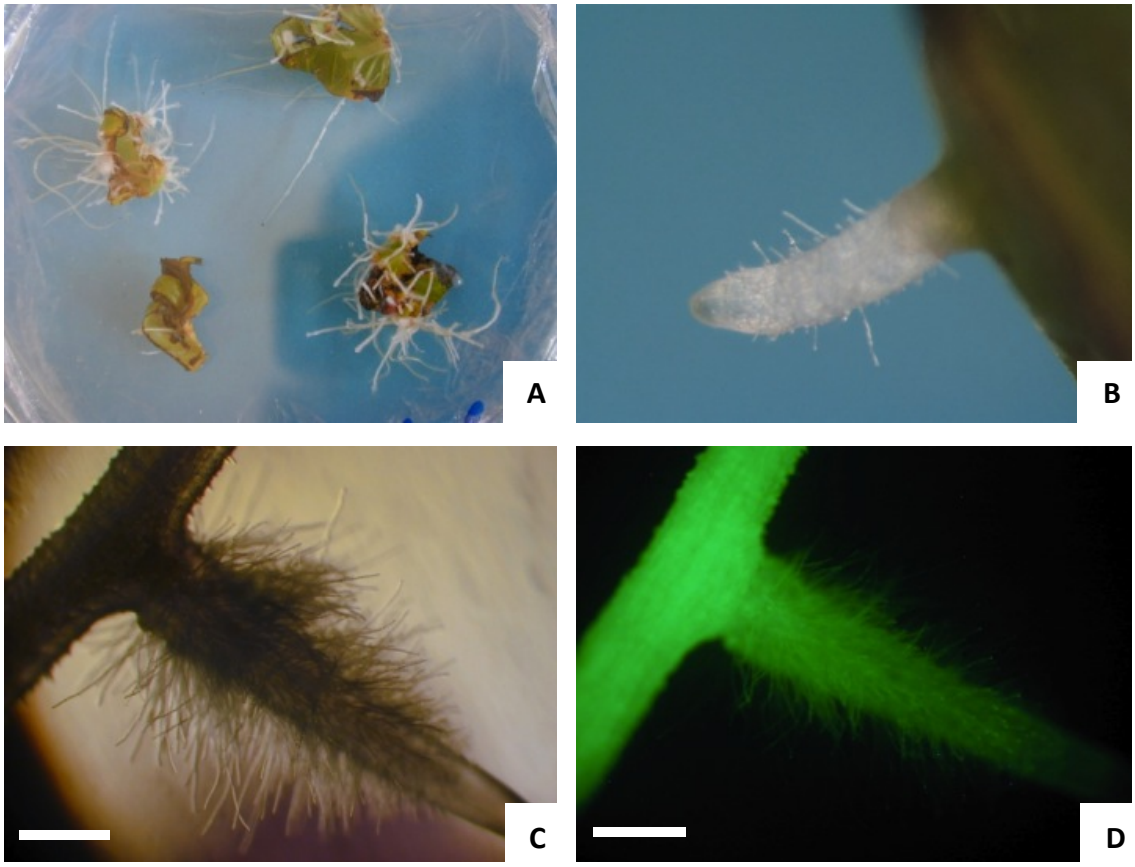


Fig 5-4 *Maesa lanceolata* hairy roots appearing at the wounded sites 2 to 4 weeks after infection (**a** – **b**); transgenic roots were selected based on GFP fluorescence (**c** – **d**). Scale bar = 1mm.

Hairy roots appeared from wounded sites 15-30 days after inoculation with *Agrobacterium rhizogenes* (Fig 5-4 a-b). Uninfected control explants did not form adventitious roots. GFP was used as a visible marker to rapidly select transformed hairy roots (Fig 5-4 c-d). Isolated hairy roots, in contrast to untransformed roots, grew autonomously in hormone free medium.

To save space in the growth rooms, stock cultures of the hairy roots were kept on solid medium in Petri dishes. When cultures needed to be grown for analysis, the roots were transferred to liquid medium in Erlenmeyers to remove the agar because agar leftovers can interfere with the measurements. For scaling up of the hairy root cultures, we also grew them in a temporary immersion bioreactor (TIB) system (Fig 5-5).

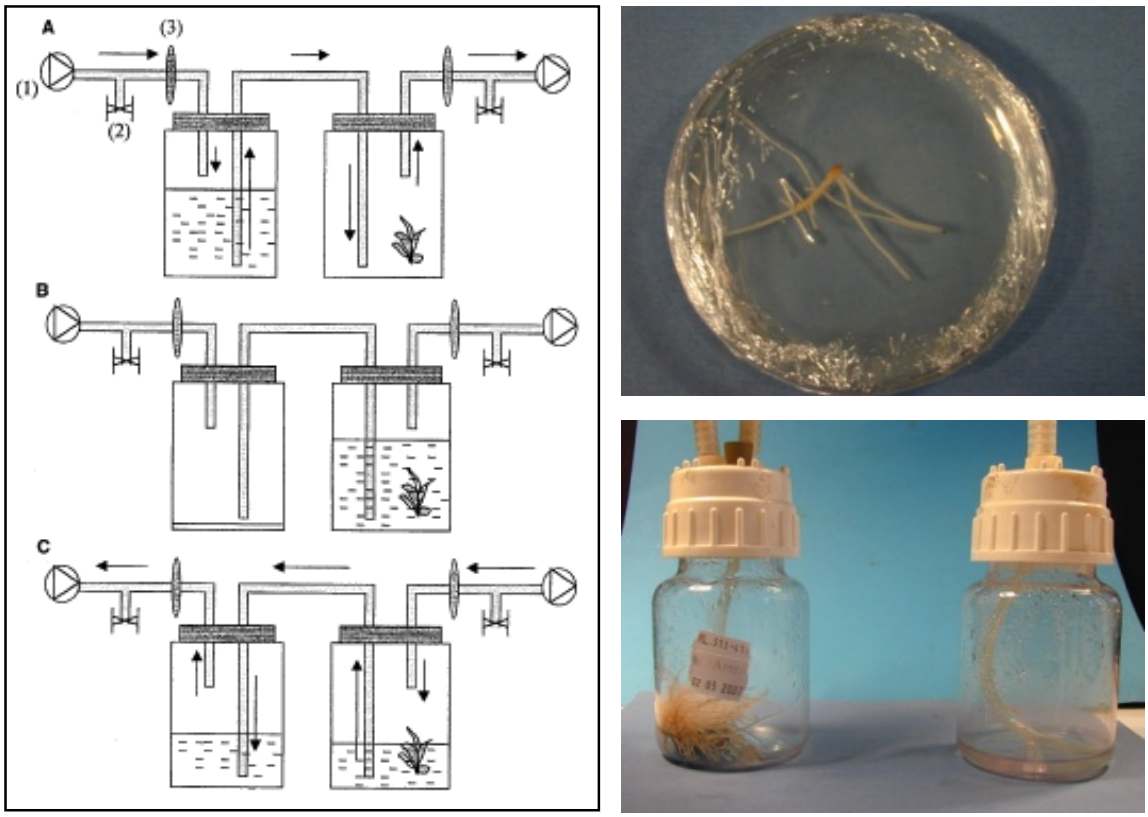


Fig 5-5 A temporary immersion bioreactor (TIB) system for *M. lanceolata* hairy roots. The system consists of two containers (one for growing the plants and a reservoir for liquid medium) that are connected with a silicon tube (**left panel, a**). At definite time points, compressed air forces the medium into the plant container, immersing the plants (**left panel, b**). After a fixed period of time, the air pressure forces the medium back into the original container (**left panel, c**) (figure adapted from (Escalona et al. 1999)). Hairy roots grown on solid medium for 1 week were used as explant for growth in TIB; after 1 month in TIB the hairy roots could be harvested for further analysis (**right panel**).

To initiate hairy root TIB cultures, the inoculum required to exceed a certain size (Fig 5-5). Single root tips did not regenerate and died upon prolonged incubation (data not shown). TIB hairy root cultures showed vigorous growth and allowed substantial biomass accumulation (Fig 5-5). Because of the lack of good RNAi silencing lines we did not further explore the TIB system for biomass production. Yet, the TIB system offers the advantages of reduced contamination risk and easy scaling up in e.g. 10 L containers for large production requirements.

5.4.2 Establishment of RNAi lines

After BLAST analysis, a selection of 41 MeJA induced genes was considered as putative saponin biosynthesis or secondary metabolite regulatory genes. 24 of these genes were cloned into an RNAi vector (pK7GWIWG2D) and 15 constructs, which contained gene sequences that were possibly involved in saponin side chain modification, were used for transformation of *M. lanceolata* leaf discs with *Agrobacterium rhizogenes.* An overview of the genes that were transformed in *M. lanceolata* is presented in Table 5-1.

Table 5-1 Putative *Maesa* saponin biosynthesis and regulator genes that were cloned in an RNAi vector and transformed in *M. lanceolata* using *Agrobacterium rhizogenes* mediated transformation

Code	Gene
ML013	Cytochrome P450-dependent fatty acid hydroxylase (CYP94A4)
ML029	Hypothetical protein
ML034	Cytochrome P450-like protein (CYP96A12)
ML041	Cytochrome P450 (CYP71A5)
ML077	P-coumaroyl shikimate 3'-hydroxylase (CYP98A3)
ML104	Cytochrome P450 (CYP71A3)
ML158	Glucosyltransferase IS5a salicylate-induced-like
ML213	Phosphoethanolamine N-methyltransferase
ML222	Alkenal reductase (AER)
ML257	Cytochrome P450 monooxygenase (CYP716A12)
ML339	Cytochrome P450 (CYP76A1)
ML385	Cytochrome P450 (CYP79D4)
ML432	Acyltransferase
ML447	Acyltransferase
ML582	Putative squalene monooxygenase

We achieved successful transformation for all 15 constructs; however, efficiency of transformation seemed to be dependent on the construct used (Table 5-2). For example, with ML013 we obtained only one well growing transgenic line, in contrast, we had 81 lines for ML582. We also noticed that a number of hairy roots stopped growing and turned brown after isolation from the leaf discs.

A number of hairy roots bearing a specific RNAi construct was analyzed for saponin production by the lab of Pharmacognosy and Pharmaceutical Analysis (University of Antwerp) through HPLC-MS. For each construct, one to 8 lines were analyzed. The saponin contents for the individual transgenic lines are shown in Table 5-2.

Table 5-2 For each RNAi constructed one or more transgenic hairy root lines could be produced; the efficiency of transformation was dependent on the RNAi construct used. The saponin content was measured (through HPLC-MS) in different lines with the same construct (1 to 8 lines were analyzed for each construct, except for ML339, ML385 and ML432). Some of the hairy root lines showed an abnormal growth phenotype. This was observed with 5 constructs; the number of abnormal lines per construct is indicated. As a control, leaves were transformed with the same vector, however, without a silencing construct.

Construct	# transgenic lines produced	Saponin content (% d.w.) in different lines analyzed								Abnormal phenotypes
		1	2	3	4	5	6	7	8	
Control	6	1.8	1.9	2.5	1.8	0.4	0.4	-	-	
ML013	1	0.6	-	-	-	-	-	-	-	
ML029	1	0.5	-	-	-	-	-	-	-	
ML034	18	0.7	0.9	0.5	0.6	0.7	-	-	-	1
ML041	18	2.1	0.3	0.6	0.4	-	-	-	-	
ML077	9	0.2	-	-	-	-	-	-	-	1
ML104	41	0.4	0.7	1.2	0.2	1.8	-	-	-	3
ML158	11	1	1	0.5	-	-	-	-	-	3
ML213	7	0.6	0.8	0.5	-	-	-	-	-	
ML222	32	0.6	0.4	1.1	1.2	1.5	1.5	1.9	0.9	5
ML257	5	0.6	1.2	2.7	-	-	-	-	-	
ML339	1	-	-	-	-	-	-	-	-	
ML385	7	-	-	-	-	-	-	-	-	
ML432	8	-	-	-	-	-	-	-	-	
ML447	4	0.5	-	-	-	-	-	-	-	
ML582	81	0.7	0.5	0.7	1	-	-	-	-	

A relatively large variation in saponin content within different control lines could be noticed; for example lines 5 and 6 produce 6 times less saponins compared to line 3. Therefore, the production of saponin in these hairy roots must be unstable or alternatively, the method for saponin concentration analysis was not sufficiently robust to allow a comparison. Keeping this variation in mind, we concluded that there were no major changes in saponin content of the RNAi lines compared to the control hairy roots.

Hairy roots bearing the RNAi construct ML432, encoding an acyltransferase, were investigated in more detail. Maesasaponins can have an acylation in three positions (R1, R2 and R3) (Chapter 1, Fig 1-4), however, the enzymes responsible for the attachment of these acyl groups to the triterpenoid skeleton are currently unknown. cDNA-AFLP analysis revealed that some acyltransferases were upregulated upon elicitation with MeJA. These might be candidates for the acylation of the maesasaponins. The total amount of saponins, as well as each individual maesasaponin, in two independent hairy root lines with the ML432 construct was analyzed (Fig 5-6).

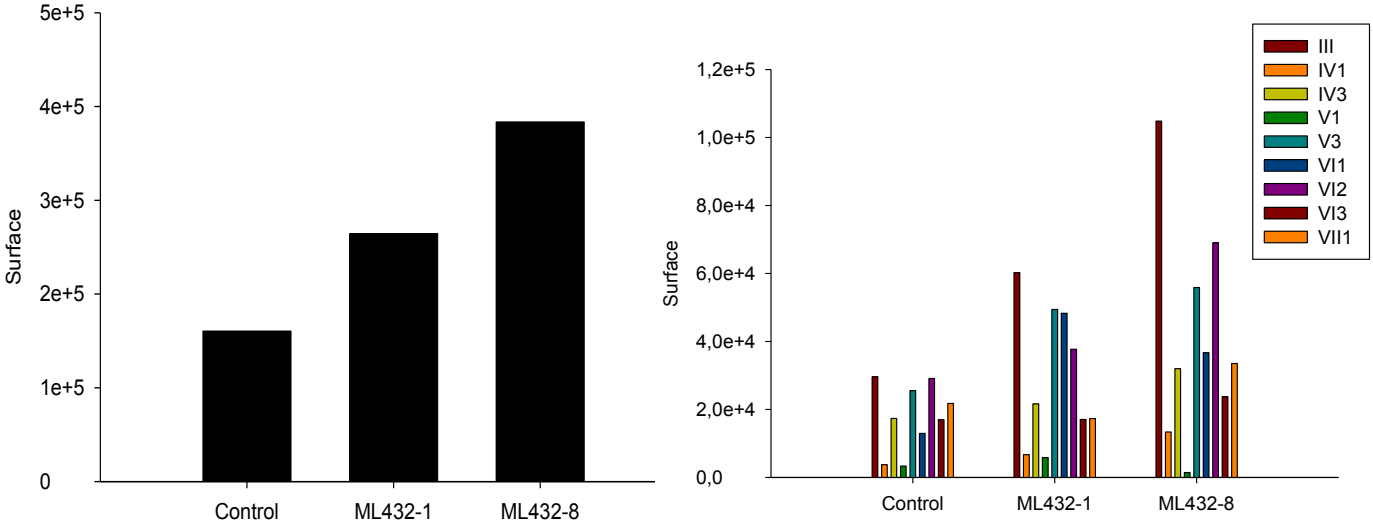


Fig 5-6 Analysis of saponin content in two independent lines of ML432. As well the total saponin content (**left histogram**) as the individual saponins (**right histogram**) was measured. Maesasaponins I, II, III_1, IV_2, V_2 and VII_2 were not detected.

In Figure 5-6 the saponin content is shown as the surface of the peaks in the chromatogram. However, the measurement is quantitative and relative differences can be deduced from the figures.

When comparing the individual saponins within the different lines, it was remarkable that 21,22 diesters (mainly maesasaponin III) were upregulated in both transgenic lines compared to the triesters (maesasaponin VI_3 and VII_1) (maesasaponin structures are represented in Figure 1-4). This could be an indication that the acyltransferase responsible for acylation of the R1 group at position 16 was (partially) silenced. Consequently, triesters should not be synthesized or only produced in lower concentrations. The two triesters maesasaponin IV_2 and V_2 were indeed not detected in the transgenic lines, however, there were also no detectable amounts present in the control lines. In addition, based on this theory, maesasaponin II should be transformed to maesasaponin I but both types of maesasaponins were not detected, neither in the controls nor in the transgenic lines. Because we cannot correlate the changes in saponin structures with the RNAi silencing it is currently not possible to draw clear-cut conclusions from these data.

Concurrent with the saponin analysis we also analyzed the RNA levels of the silenced genes using quantitative PCR (qPCR). Results for three independent control lines (ML000.2, ML000.6, ML000.7) and three lines transformed with construct ML034 (ML034.1, ML034.3, ML034.5) are represented in Figure 5-7. All six lines were analyzed with three primer pairs; two household genes (a proteasome component and β-tubulin 6) and the ML032 gene (a cytochrome P450-like protein).

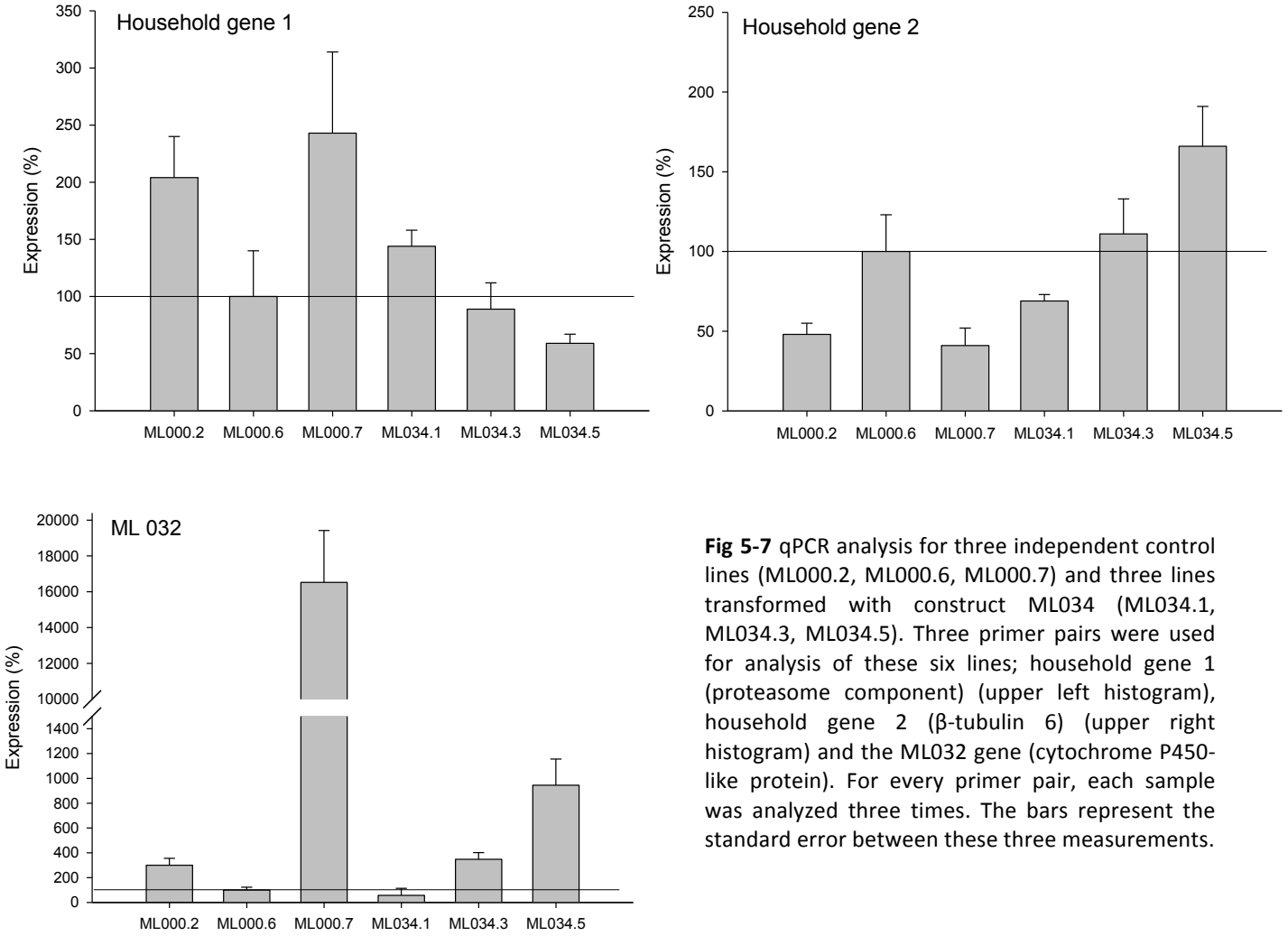


Fig 5-7 qPCR analysis for three independent control lines (ML000.2, ML000.6, ML000.7) and three lines transformed with construct ML034 (ML034.1, ML034.3, ML034.5). Three primer pairs were used for analysis of these six lines; household gene 1 (proteasome component) (upper left histogram), household gene 2 (β-tubulin 6) (upper right histogram) and the ML032 gene (cytochrome P450-like protein). For every primer pair, each sample was analyzed three times. The bars represent the standard error between these three measurements.

From figure 5-7 it can be deduced that the RNA levels showed large variations both in the control as in the RNAi lines. We have also tested 3 additional primer pairs (β-amyrin synthase, a putative squalene monooxygenase and a carbonic anhydrase) and 9 additional RNAi hairy root lines (5 independent ML034 lines and 4 ML432 lines), though, we never obtained reliable results because of very large variations with

all lines and primers tested. Further optimization of sample preparation and perhaps also the qPCR primers would be required in order to obtain more reliable data. Because of the technical difficulties in obtaining sound RNA expression data, we reasoned that the overexpression of putative saponin biosynthesis genes had the potential to cause more profound effects on both the saponin production as well as target gene expression. After all, the recording of an increase in RNA levels is more readily detected than a potentially partial reduction through silencing. Hence we focused on an overexpression approach. Different putative saponin biosynthesis genes of other species were transformed in *Maesa lanceolata* in order to change the saponin production. This part of the Combiplan was performed by other researchers; therefore the results of these experiments, as well as the results in *Medicago truncatula*, are not included here. However, they will be considered in the Discussion part of this chapter.

5.4.3 Hairy roots with abnormal phenotypes

Some lines of hairy roots bearing the constructs ML158, ML104, ML077, ML222 and ML034 displayed a phenotype that clearly differed from that of control hairy roots (Table 5-2, Fig 5-7). For ML158, 3 out of the 11 independent lines had roots that were more thick and flat than the control hairy roots. In addition, the oldest parts of the roots became brown and sometimes started to dedifferentiate to callus. Hairy roots from 3 out of the 41 ML104 lines did not seem to elongate but did form numerous side roots which led to a shape that resembled a 'sea urchin'. One line out of the 9 bearing the ML077 construct showed roots that were very hard and thick. Emerging lateral roots were also thick and hard so it looked like the main root had bulges over the whole length but did not show the presence of root hairs. For the construct ML222, 5 lines out of the 32 and for ML034, 1 line out of the 18, produced thick and 'fluffy' hairy roots. These roots did also not elongate but made many side roots. The reason for this abnormal morphologies was not known, it was notable, though, that the phenotypes of ML222 and ML034 resembled the phenotypes of control hairy roots treated with auxin (Fig 5-8).

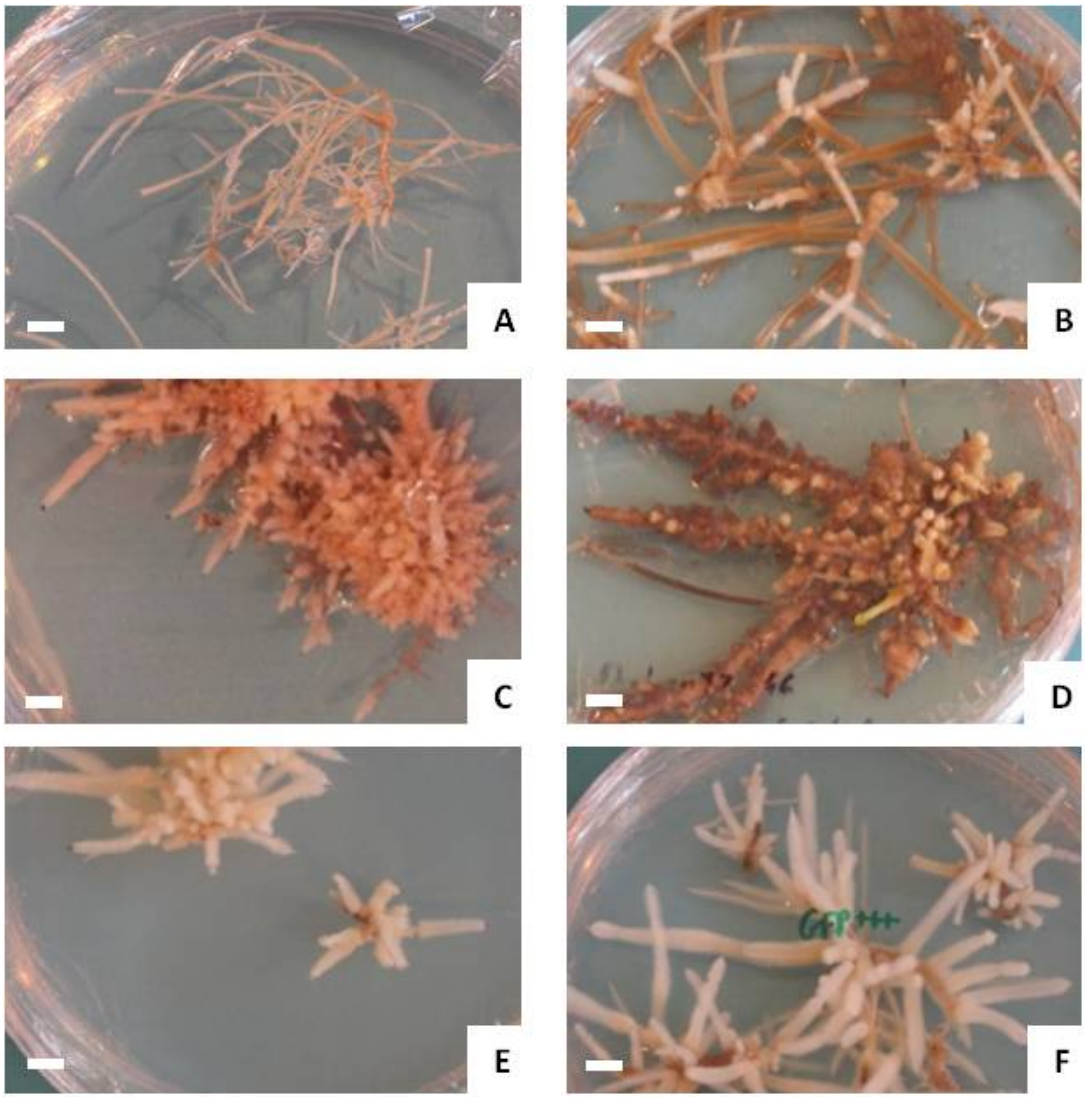


Fig 5-8 Some of the hairy roots, transformed with RNAi constructs showed phenotypes that strikingly differed from the control hairy roots (**a**). Abnormal phenotypes were observed with the following constructs; ML158 (**b**), ML104 (**c**), ML077 (**d**), ML222 (**e**), ML034 (**f**). In general, hairy root lines with abnormal phenotypes are thicker compared to control lines. They also don't elongate as much as the control roots and they make numerous side roots and root hairs, which gives them an overall 'fluffy' appearance. Only abnormal lines of the construct ML077 (d) do not have root hairs; these roots are very thick and hard and have 'bulges' over the whole length. Scale bar = 0.5 cm.

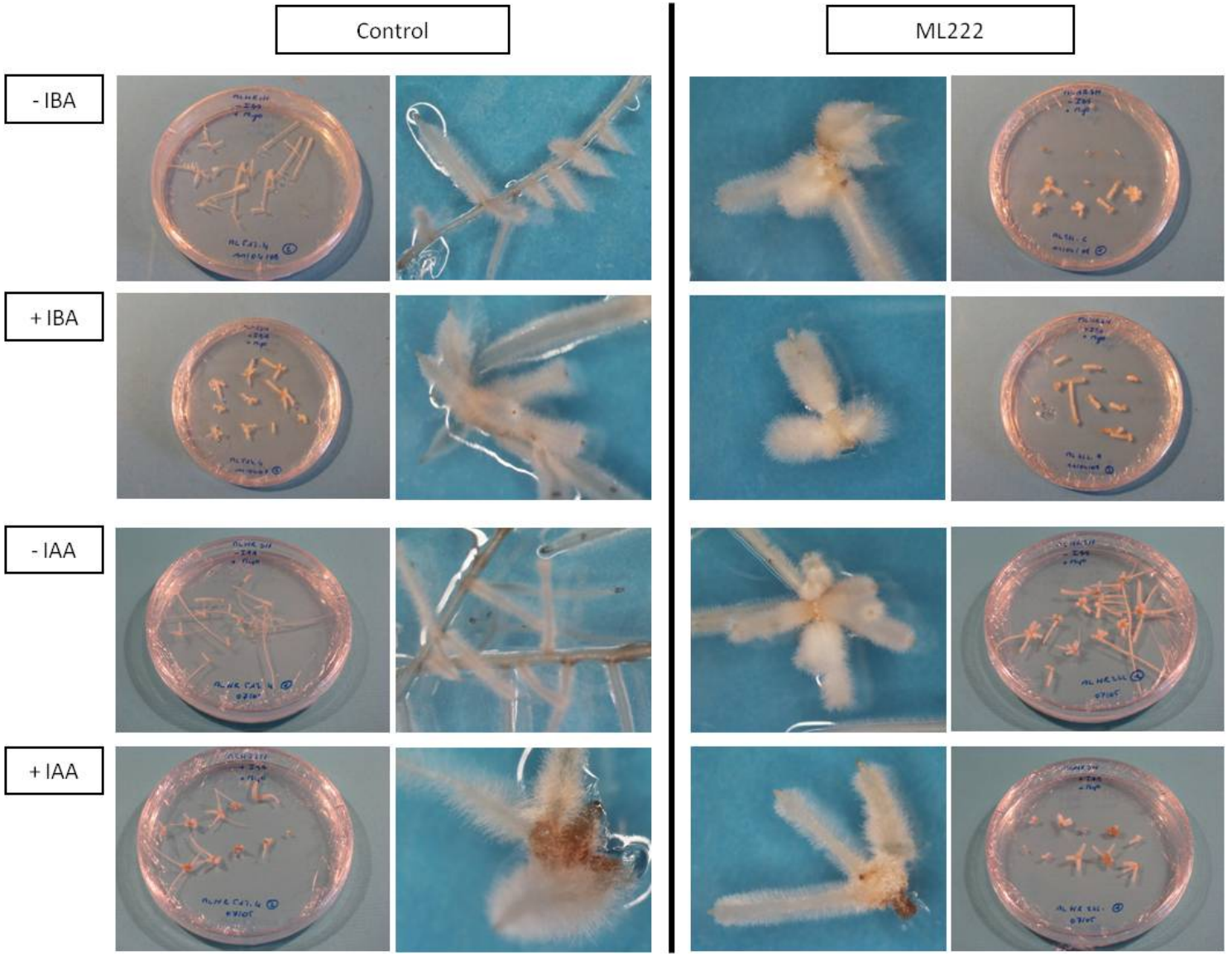


Fig 5-9 The abnormal phenotypes of hairy roots with the construct ML222 resemble the phenotype of control hairy roots treated with 10µM IBA or 10µM IAA. ML222 roots did not show morphological alterations in response to the applied hormones.

5.5 Discussion

In this chapter, we describe the establishment of a protocol for genetic transformation of the medicinal plant *Maesa lanceolata*. A method was also developed for efficient culturing and upscaling of the resulting hairy roots. These technical tools were developed within the framework of the Combiplan project. The **Combiplan** or **COMbinatorial BIosynthesis in PLANts** project conceived a strategy to identify and exploit saponin biosynthesis genes based on the high-throughput selection and testing of genes. Moreover, genes of different species will be transformed in different species to create diversity in secondary metabolism. The concept is that an enzyme with a specific substrate isolated from one plant might encounter new but related substrates when introduced in another plant (Oksman-Caldentey and Inze 2004). To obtain proof of concept, six target plants were chosen that all produced triterpene saponins with medicinal properties, with *Medicago truncatula* and *Maesa lanceolata* being the main species used.

For *Maesa lanceolata* a transformation protocol was not available, thus, an efficient method for transformation using *Agrobacterium rhizogenes* was first established. Transgenic hairy roots were induced on *Maesa* leaf discs and were, after isolation, grown on culture medium without the need of exogenous hormones. The growth on hormone-free medium is one of the advantages of hairy roots over normal roots (Giri and Narasu 2000). Hairy roots are more differentiated than cell cultures, therefore, they are genetically and biochemically more stable and consequently offer a promising alternative for the production of important metabolites (Georgiev et al. 2007). Many examples of hairy root cultures producing secondary metabolites with medicinal activity are available, for example production of artemisinin in *Artemisia annua* hairy roots (Weathers et al. 2005), camptothecin in *Ophiorrhiza pumila* and *Camptotheca acuminata* (Saito et al. 2001), scopolamine and hysoscyamine in *Datura innoxia* (Dechaux and Boitel-Conti 2005) and taxol in *Taxus brevifolia* (Huang et al. 1997). Using TLC analysis, we could also detect saponins *Maesa lanceolata* hairy roots that were qualitatively and quantitatively comparable to normal roots of greenhouse grown and *in vitro* grown plants (Chapter 4).

Maesa lanceolata hairy roots could easily be upscaled in a temporary immersion bioreactor (TIB). An automated bioreactor system enables a constant supply of nutrients and aeration to plants without the use of sophisticated or expensive technology. In addition, growing plant material in TIB requires less handling of the plant material and consequently results in less risk on contamination. The system applied for *Maesa lanceolata* hairy roots consisted of two culture vessels, one contained the hairy roots and the

other one contained the liquid medium. The two containers were connected with a silicon tube and every 3 hours, compressed air forced the medium into the plant container, immersing the hairy roots. In our lab, the TIB system was applied for the growth of plantain shoots and resulted in a 2 fold higher multiplication rate than the growth in semi-solid medium (Roels et al. 2005). To the best of our knowledge, this is the first time that the successful growth of hairy roots in such a bioreactor system is reported.

One of the aims of the Combiplan was to create novel maesasaponin molecules. For this purpose, two approaches were employed. For homologous transformation, i.e. using *Maesa* genes, the entire open reading frame of the corresponding cDNA-AFLP tag would be introduced in RNAi cassettes to knock down certain metabolic conversions leading to particular natural substitutions or modifications of the saponin skeleton that might have certain effects on the activity or toxicity of the maesasaponins. However, none of the silencing constructs tested in *Maesa lanceolata* changed the saponin production. In addition, we were not able to proof the gene silencing with quantitative PCR because of technical problems. Therefore, the focus was shifted towards the second method that was developed to alter the saponin production in *Maesa*; namely overexpression of heterologous genes. This might allow the introduction of novel substitution groups or changing of the skeleton of maesasaponins and consequently, novel molecules with potential superior properties may be created. In total 49 gene constructs, from 4 different plant species, were transformed in *Maesa*. Genes were isolated from *Bupleurum falcatum*, *Medicago truncatula*, *Panax ginseng* and *Glycyrrhiza glabra.* All four species produce structurally related triterpene saponins, however, with different structural features linked to different biological activities. For 25 constructs overexpression was confirmed in different hairy root lines (Faizal et al. *unpublished results*). Ten of these confirmed overexpression constructs are currently being analysed with HPLC-MS techniques (more specifically UPLC/ICR-FT-MS or Ultrahigh Performance Liquid Chromatography/Ion Cyclotron Resonance – Fourier Transform – Mass Spectrometry), for every construct 5 repeats of 3 independent lines are investigated. Preliminary results show that there are potentially novel saponins present in 5 out of the 10 investigated overexpression lines. For these 5 constructs, peaks were found in the chromatogram of all 15 repeats that were not observed in the control lines. Further research is necessary to identify these peaks and learn more about the putative new saponins that have been formed.

Although *Maesa lanceolata* was initially the preferred target species for transgene introduction, *Medicago truncatula* evolved as a more agile complement of the combinatorial biosynthesis program.

The experiments with *Medicago truncatula* were performed in the Secondary Metabolites Group of the Plant Systems Biology department (VIB). For *Medicago,* silencing could be confirmed for 12 genes using qPCR, additionally, overexpression of 60 heterologous genes was proved with RT-PCR. All confirmed hairy root lines were upscaled for metabolite profiling. Also for *Medicago,* the RNAi approach did not prove successful for changing the saponin mixture. In contrast, five overexpression constructs were analysed in more detail with UPLC/ICR-FT-MS and for two constructs, a dammarenediol synthase from *Panax ginseng* and a cytochrome P450 from *Bupleurum falcatum*, new compounds were found. Further comparative studies revealed that some of the compounds that were present in the transgenic *Medicago* hairy roots were not present in *Medicago, Panax* or *Bupleurum* control roots. Currently more in depth analyses are being performed to determine the identity of these novel molecules (Pollier et al. *unpublished results*).

Normal roots generally respond to auxin treatment through an increased lateral root formation and a reduced elongation. Remarkably, this phenotype was noticed in some hairy root lines without the application of exogenous auxins. Multiple lines from 5 out of the 15 transformed antisense constructs had a morphology that was different from the control hairy roots lines; many more lateral roots emerged and elongation was strongly diminished. In addition, the roots were very thick and in some cases no root hairs were formed. It is unlikely that these phenotypes are a direct effect of the silencing in the hairy roots because for none of the lines could we find a strong correlation between the presence of the construct, by means of GFP fluorescence, and the root phenotype. The morphological aspects of the roots resemble that of the short thick root phenotype that has been described for *A. rhizogenes* transformation events. Two primary groups of the Ri (root inducing) genes are involved in the root induction process: the *rol* genes in the T_L-region and *aux* genes in the T_R-region (White et al. 1985). For *Cucumis sativus* hairy roots, similar atypical hairy root phenotypes were observed. Further investigations proved that mainly the *aux* genes from the T_R-region play a role in determining the short thick root phenotype. More specifically, transcript analysis showed a correlation between higher expression of *aux2* and short thick roots. There was no correlation found between copy number and phenotype (Amselem and Tepfer 1992). Transformations in both *Maesa* and *Cucumis* were performed with an agropine-type *Agrobacterium rhizogenes* strain. *Pisum sativum* was transformed with complete T-DNA, T_L-fragments and T_R-fragments, also using an agropine-type strain. Results showed that T_L-roots were long and thin and T_R-roots were short and ramified. Roots with both T_L- and T_R-fragments had an intermediate, typical phenotype (Prinsen et al. 1992). Agropine-type *A. rhizogenes* strains can both

increase the sensitivity to auxin and increase the endogenous auxin levels in hairy roots. However, comparison of *Hyoscyamus muticus* normal roots and hairy roots showed comparatively low levels of free IAA. So it appears that control of growth and development in hairy roots involves factors other than IAA overproduction. However, due to the complexity of the system, many questions remain unanswered (Biondi et al. 1997). In contrast to the previous studies in *Cucumis* and *Pisum*, the phenotypic differences that were found in *Daucus carota* hairy roots could not be strictly correlated to the transformation events (Guivarc'h et al. 1999). Without further investigations it is not possible to explain the atypical hairy root phenotypes in *Maesa lanceolata* on a molecular level. However, based on literature searches, we believe that the phenotypes are most likely caused by non-specific transformation events, similar to the asymmetrical insertion of T_L- and T_R-regions.

The primary objective of this project was to establish a combinatorial biosynthesis platform in plants that allows the semi-rational combinatorial engineering of the biosynthesis of existing and novel secondary metabolites in plant tissue cultures. To obtain proof of concept, a high-throughput cDNA-AFLP method was developed to identify putative triterpene saponin biosynthesis genes. This method already proved successful for the elucidation of alkaloid biosynthesis genes in tobacco Bright yellow cell cultures (Goossens et al. 2003). Putative saponin biosynthesis and regulator genes were cloned in RNAi and OE vectors and the constructs were transformed into *Maesa* and *Medicago* hairy roots. However, the RNAi approach was inadequate to obtain proof of concept for the Combiplan. As well for *Maesa* as for *Medicago* no changes in saponin production were observed after silencing endogenous genes. In contrast, the overexpression of heterologous genes did prove to be more successful. Although more detailed studies are still ongoing, we can already state that through the overexpression of heterologous genes, identified through cDNA-AFLP analysis, novel compounds are produced as well in *Maesa* as in *Medicago* hairy roots. These recent findings are explicit evidence for combinatorial biosynthesis in plants.

5.6 Materials and methods

5.6.1 Plant material

Maesa lanceolata seeds were collected in Moshi, Tanzania by Frank Mbago (Department of Botany, University of Dar-Es-Salaam). The method for sterilization and *in vitro* cultivation is described in the Materials and methods section of Chapter 2.

5.6.2 Supporting work

Elicitation, AFLP, gene sequencing and selection

Initial experiments of the Combiplan were performed by the Secondary Metabolites Group from the Department of Plant Systems Biology (VIB).
For elicitation, *in vitro* shoots of *M. lanceolata* were sprayed with 500µM methyl jasmonate (MeJA) or ethanol (as a control) until run-off. Leaves were harvested 0, 0.5, 1, 2, 4, 8, 24 and 48 hours after elicitor treatment. After sampling, total RNA was prepared with TRIzol (Invitrogen, Carlsbad, CA) and reverse transcribed to double-stranded cDNA as described (Vuylsteke et al. 2007). Upon appropriate sample preparation, cDNA-AFLP based transcript profiling was performed as described with all 128 possible BstYI+1/MseI+2 primer combinations (Rischer et al. 2006; Vuylsteke et al. 2007). Gel images were analyzed with the AFLP-QUANTARPRO software (Keygene, Wageningen, The Netherlands) allowing accurate quantification of the band intensities. The intensity of all individual bands was determined and the obtained raw expression data were corrected for lane variations (due to PCR or loading differences) by dividing the raw data by a correction factor. The correction factor was calculated by dividing the sum of the expression levels of all fragments within one lane by the highest sum of all lanes within a primer combination. Subsequently, the standard deviation (SD) and the average were calculated for each individual band. Individual gene expression profiles were variance normalized by subtracting the calculated average from each individual data point, after which the obtained value was divided by the SD. A coefficient of variation (CV) was obtained by dividing the SD by the calculated average. Gene tags displaying expression values with a CV≥0.5 were considered as differentially expressed. Based on this cut-off value, together with visual inspection of cDNA-AFLP gels, differentially expressed gene tags were selected for further analysis. Cluster analysis, sequencing and BLAST analysis were performed as described by Rischer et al. (2006).

HPLC-MS for saponin analysis

All saponin analyses were performed in the Lab of Pharmacognosy and Pharmaceutical Analysis (University of Antwerp). The protocol for *Maesa* sample preparation and HPLC-MS is described in Theunis et al. (2007).

5.6.3 *Maesa lanceolata* hairy root induction and culture

Maesa lanceolata hairy roots were induced through *Agrobacterium rhizogenes* (strain LBA 9402/12) transformation on leaf discs. The *Agrobacterium* strain was transformed with the pK7WG2D or pK7GWIWG2D plasmid (Fig 5-9). *Agrobacteria* were transferred from a glycerol stock to 3ml of liquid YEB medium. This medium contained antibiotics (100mg/l rifampicin (Sigma), 100mg/l spectinomycin (Sigma) and 300mg/l streptomycin (BDH)) and consisted of 5g/l beef extract (Sigma), 1g/l yeast extract (Duchefa, The Netherlands), 5g/l peptone (Duchefa, The Netherlands), 0.15M sucrose and 2ml/l 1M $MgSO_2$. The bacterial cultures were incubated on a rotary shaker (220 rpm) at 28°C. After 48 hours 20µl of this bacterial preculture is brought into 5ml of fresh YEB medium with the same antibiotic concentrations as mentioned before. The bacterial cultures were again incubated on a rotary shaker (220 rpm) at 28°C. 48 hours later, these cultures were used for the transformation. For the selection of the transgenic material, GFP was used as a visible marker.

For hairy root induction, *in vitro* leaf material was wounded and co-cultivated with the *Agrobacteria*. The co-cultivation medium consisted of 1x MS salts with vitamins supplemented with 0.8% (w/v) agar, 2% (w/v) sucrose and 4 µM Benzyladenine (BA) (Sigma). After 3 days, the leaves were transferred to cultivation medium comprising 1x MS salts with vitamins, 0.8% (w/v) agar, 2% (w/v) sucrose and 500 mg/l cefotaxime (Duchefa, The Netherlands) to arrest bacterial growth. Hairy roots were isolated from the leaf material after 15-30 days and were placed on solid SH medium including vitamins and supplemented with 0.8% (w/v) agar and 3% (w/v) sucrose. The cultures were incubated in the dark at 25°C and were subcultured monthly.

For saponin analysis through HPLC-MS, hairy roots were grown in liquid cultures. Hairy roots were transferred to Erlenmeyers with 10 ml of the SH culture medium described above. For upscaling the hairy roots, they were cultured in temporary immersion bioreactors. Hairy roots were put in one container and 25 ml of the liquid culture medium was brought in the other container. The medium was transferred from the medium vessel to the hairy root vessel every 3 hours for 4 minutes.

To mimic the phenotypes of some RNAi lines, control hairy roots and ML222 hairy roots were transferred to solid medium supplemented with 10 µM IAA (Fluka) or 10 µM IBA (Fluka).

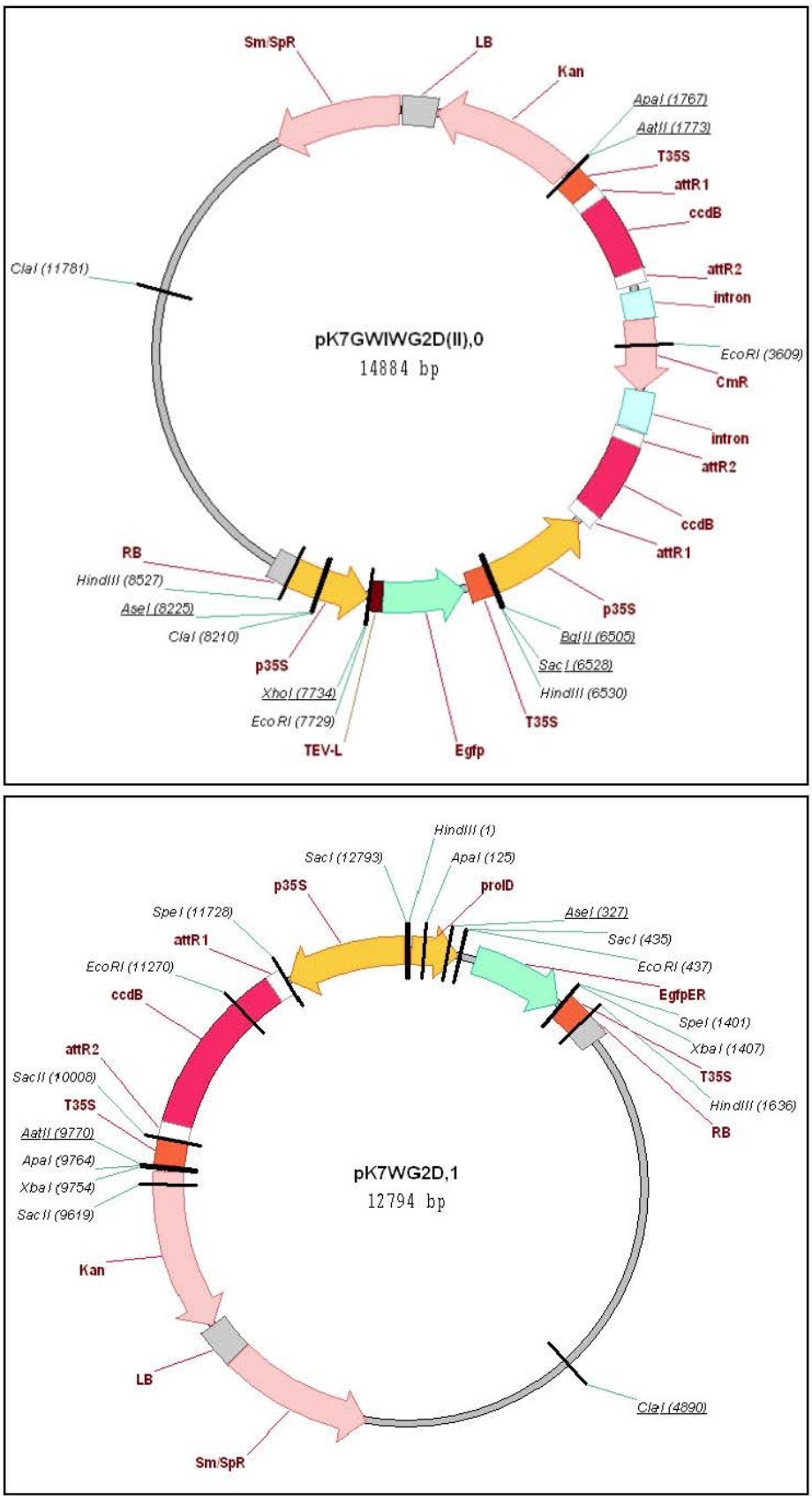


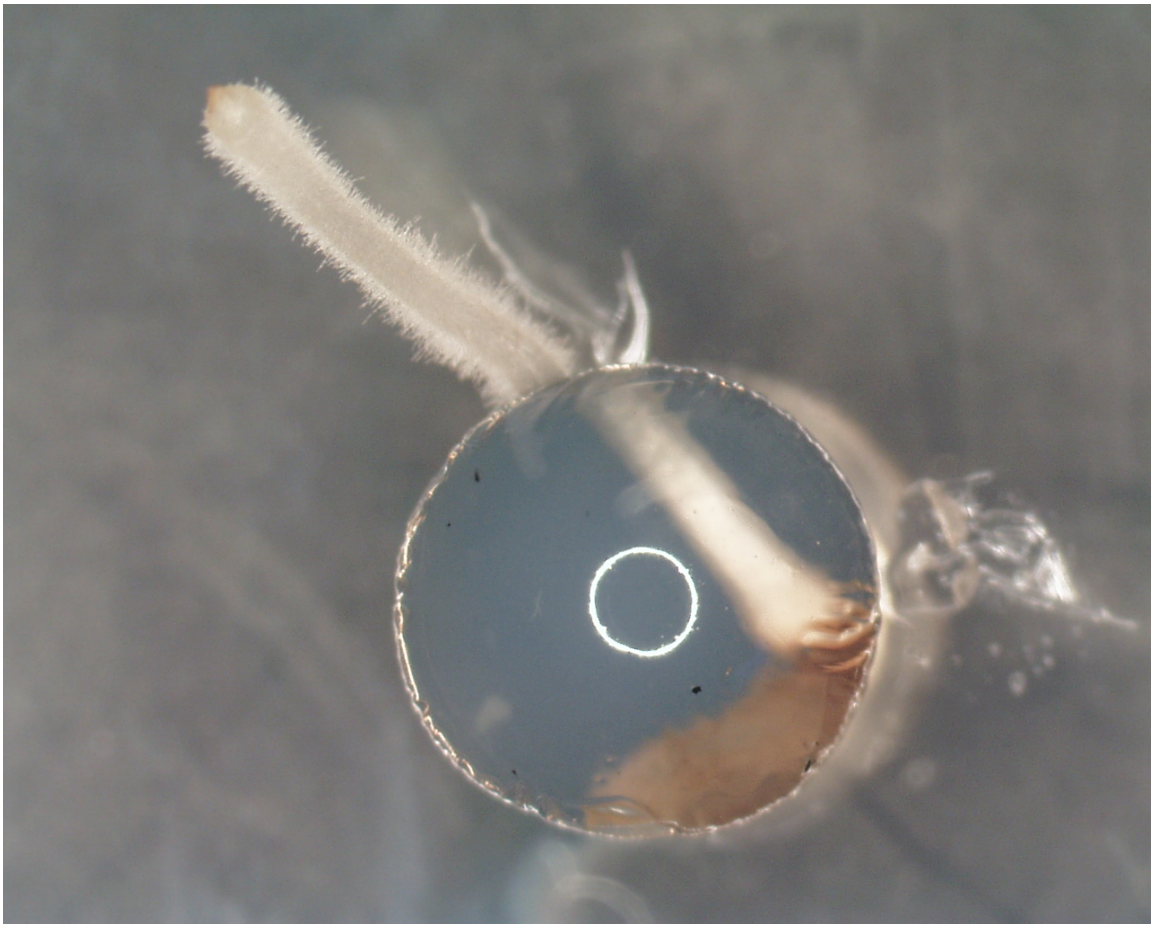
Fig 5-10 Vector backbones for RNAi silencing (**pK7GWIWG2D**) and overexpression (**pK7WG2D**) analysis in *Maesa lanceolata* hairy roots

5.6.4 Quantitative PCR (qPCR)

qPCR experiments were performed in the Secondary Metabolites Group (VIB). Total RNA was prepared with TRIzol (Invitrogen, Carlsbad, CA) and reverse transcribed to cDNA as described (Vuylsteke et al. 2007). A robot (controlled by Xiril Software) was used to transfer sample cDNA, primers and SYBRGreen Mastermix (Roche Diagnostics) to a multiwell plate (384 wells, Roche) according to the manufacturer's instructions. Afterwards the plates were mounted into the PCR device (LightCycler®480). Roche software (https//www.roche-applied-science.com) was subsequently used to collect and analyze qPCR data. Through specific primers (based on full gene sequences) the following genes were amplified; *ML003* = *carbonic anhydrase*, *ML746* = *proteasome component*, *ML753* = *β-tubulin 6*, *ML034* = *cytochrome P450-like protein*, *ML582* = *squalene epoxidase* and *MLBAS* = *β-amyrin synthase*

CHAPTER 6

CRYOPRESERVATION OF MAESA LANCEOLATA AND MEDICAGO TRUNCATULA HAIRY ROOTS



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6.1 Abstract

To study the production of secondary metabolites of *Maesa lanceolata* and *Medicago truncatula*, hairy root cultures of both plant species were established (Chapter 5). Because maintenance of large numbers of cultures is laborious and costly, we developed a cryopreservation protocol and stored different isolated lines over time. Using encapsulation-dehydration, high survival rates were observed for both *Maesa* and *Medicago* hairy roots. Root tips were isolated and encapsulated in calcium-alginate beads, containing 0.1M sucrose. The encapsulated hairy roots were precultured for 3 days using basal medium containing high sucrose concentrations. *Medicago* root tip growth during the preculturing time lead to unwanted outgrowth which could be tempered by addition of plant growth inhibitors. After preculturing, the beads were dehydrated in the air flow of a laminar flow until 35-40% of the initial bead weight was reached. Dehydrated beads were plunged into liquid nitrogen and after different storage times thawed in a water bath at 40°C. The survival rates were 90% for *Maesa* and 53% for *Medicago*, which are sufficient to allow implementation in large storage experimental set-ups.

6.2 Introduction

To genetically manipulate and evaluate the production of saponins in *Maesa lanceolata* and *Medicago truncatula*, hairy roots were established by infecting leaf discs and seedlings with *Agrobacterium rhizogenes*. Hairy roots are more complex structures compared to cell suspensions and the presence of differentiated cells and tissue has been proposed to ascertain a higher genetic stability (Hu and Du 2006). Other advantages of hairy roots include rapid biomass accumulation, typically accompanied with a high production of secondary metabolites and the possibility for upscaling in specialized bioreactors (Georgiev et al. 2007; Kim et al. 2002).

The down side of the use of *in vitro* hairy root cultures is that the maintenance is labour intensive and involves culture handling with a high risk of microbial contamination and subsequent loss of original cultures (Grout 1995). Also, prolonged subculturing may affect quantitative or qualitative aspects of secondary metabolite production due to the accumulation of (epi)genetic mutations. The conservation of original cultures using cryopreservation technology can avoid these problems. Storing samples in liquid nitrogen eliminates the need for periodic subculturing and reduces the risk of accumulation of somaclonal variation (Teoh et al. 1996).

Three main techniques can roughly be distinguished for plant cryopreservation; vitrification, encapsulation-dehydration and controlled rate freezing.

Despite the progress that has been made in the last decade concerning cryopreservation of plant material, there are only few reports on cryopreservation of hairy root cultures. Vitrification protocols are described for hairy roots of *Atropa belladonna* (Touno et al. 2006), *Panax ginseng* (Yoshimatsu et al. 1996) and *Angelica acutiloba* (Yoshimatsu 2000), while the encapsulation-dehydration method was used for *Vinca minor* (Hirata et al. 2002) and *Armoracia rusticana* (horseradish) hairy roots (Hirata et al. 1998). One single report describes a slow freezing method for *Artemisia annua* hairy roots (Teoh et al. 1996).

The present research was carried out to establish a robust method for the cryopreservation of hairy roots from saponin producing plants. Vitrification and encapsulation-dehydration procedures were tested and for the latter technique, preculture conditions and dehydration times were optimized.

6.3 Results

6.3.1 Hairy root cultures of *Maesa lanceolata* and *Medicago truncatula*

Maesa lanceolata and *Medicago truncatula* hairy roots appeared from wounded sites 15-30 days after inoculation with *Agrobacterium rhizogenes* strain LBA 9402/12. Uninfected control explants did not form adventitious roots. GFP was used as a visible marker to select transformed hairy roots. Isolated hairy roots, in contrast to untransformed roots, grew autonomously in hormone free medium. Figures 6-1 a-d represent the hairy root cultures of *Maesa* and *Medicago,* grown in the dark at 25°C on solid and in liquid medium.

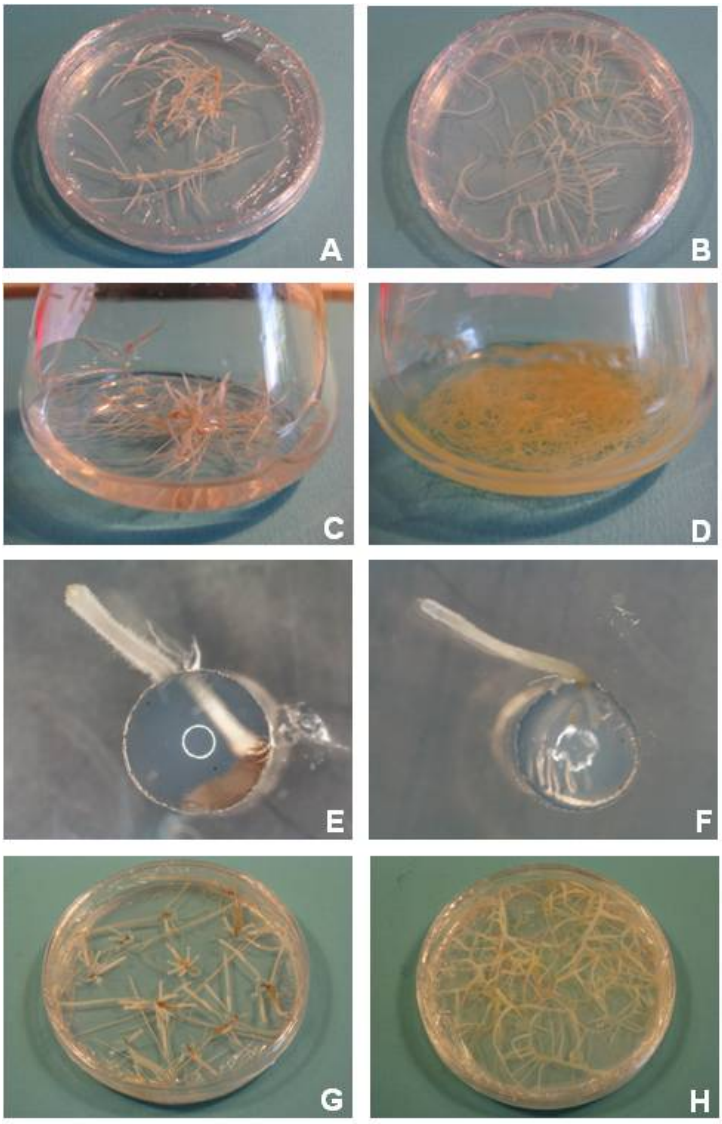


Fig 6-1 Morphological features of *Maesa lanceolata* and *Medicago truncatula* hairy roots before and after freezing. (**a-b**) Hairy roots of *Maesa* and *Medicago* on solid medium at 25°C in the dark. (**c-d**) Hairy roots of *Maesa* and *Medicago* on liquid medium at 25°C in the dark, 120rpm. (**e-f**) Hairy roots of *Maesa* and *Medicago* growing out of the calcium-alginate beads, two weeks after thawing. (**g-h**) Hairy roots of *Maesa* and *Medicago* after cryopreservation, allowed to recover for four weeks.

Hairy roots of *Medicago* grew much faster compared to *Maesa* hairy roots. *Medicago* hairy roots also appeared more fragile, thinner and more yellow than those of *Maesa.* In general, both hairy root cultures grew well on solid and in liquid medium. For the cryopreservation experiments we used hairy roots growing on plates since these were easier to handle.

6.3.2 Cytotoxicity of vitrification solution

One of the major disadvantages of the vitrification protocols using PVS2 is its cytotoxicity (Volk et al. 2006; Xue et al. 2008). PVS2 toxicity tests with *Maesa lanceolata* and *Medicago truncatula* hairy roots were preformed prior to freezing. Treatment of *Maesa lanceolata* root tips with PVS2 solution for more than 2 minutes strongly reduced their survival (Fig 6-2A).

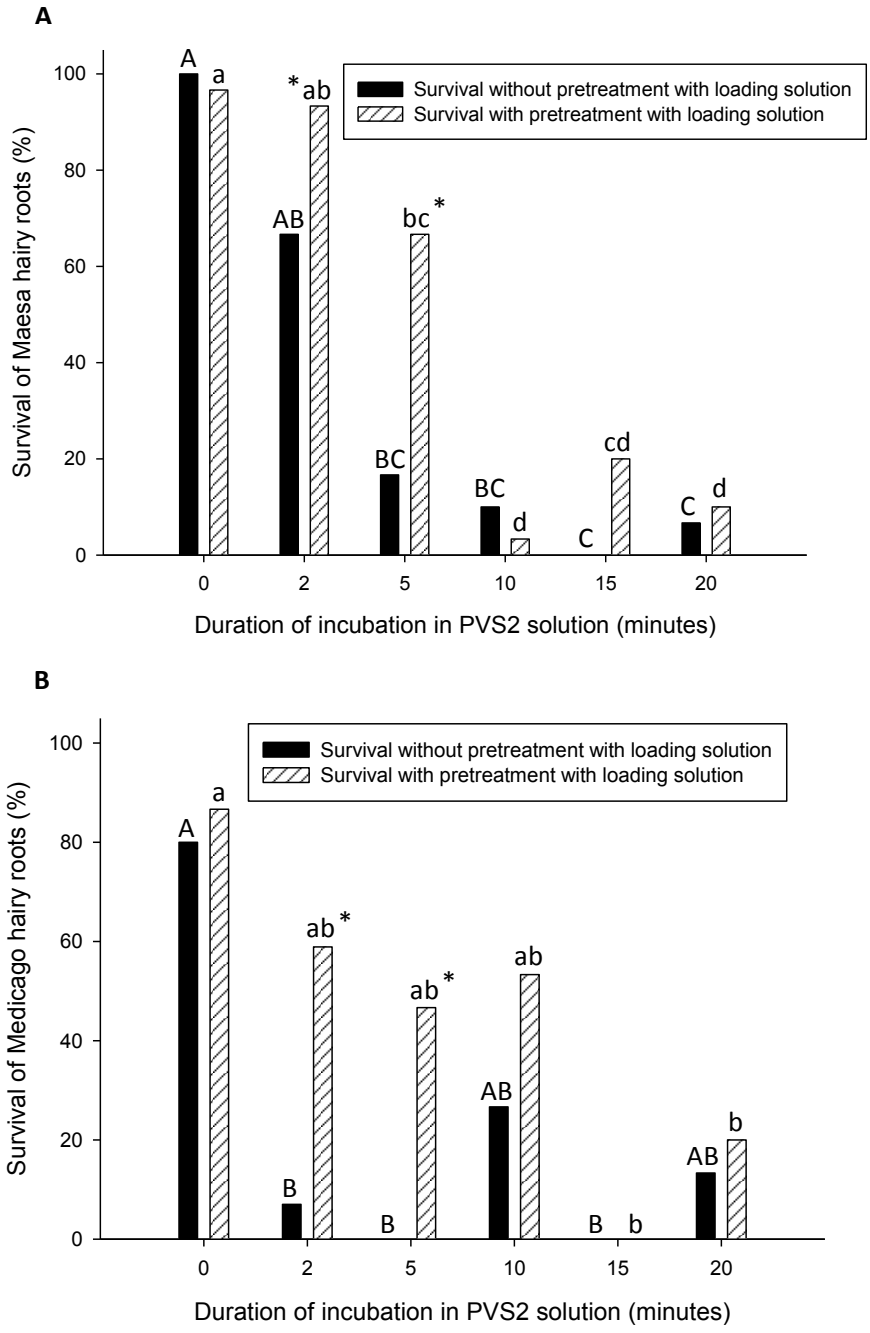


Fig 6-2 The effect of PVS2 treatment on hairy roots of (histogram **a**) *Maesa lanceolata* and (histogram **b**) *Medicago truncatula*, prior to freezing. Different incubation times in PVS2 solution were tested. Also the influence of 10 minutes pretreatment with loading solution was investigated for both *Maesa* and *Medicago*.
Each experiment consisted of three repeats with at least 10 hairy roots in each repeat. Different letters indicate significant differences ($P < 0.05$), * = significant difference ($P < 0.05$) between two different treatments on the same time point; according to non parametric ANOVA.

After 5 minutes of PVS2 treatment the survival rate was reduced to 16%. Pretreatment of the roots with loading solution (LS) had a clearly positive effect on viability of the *Maesa* hairy roots, maintaining the survival rate at 66% after 5 minutes of PVS2 treatment. Longer incubation times lead to a strong decrease in viability for both root tips treated with loading solution and without loading solution (Fig 6-2a).

Medicago hairy roots also proved to be very sensitive to PVS2 treatment. However, as for *Maesa* hairy roots, pretreatment with loading solution had a positive effect. When PVS2 was directly applied to the *Medicago* hairy roots, survival decreased significantly after 2 minutes. When the roots were pretreated with loading solution, survival was still 53%. However, survival decreased to 0% after 15 minutes of incubation in PVS2 solution (Fig 6-2b).

Microscopic inspection of root tips, using trypan blue staining, showed that after 15 min incubation time there were no more living cells in the *Medicago* and *Maesa* root tips. The high toxicity of the PVS2 solution to *Maesa* and *Medicago* root tips, disallowed recovery of the material after liquid nitrogen freezing was performed. For the freezing experiments, different PVS2 incubation times were tested (0, 2, 5, 10, 15 and 20 minutes) and part of the samples was treated with loading solution prior to incubation in PVS2 solution. Neither of these conditions led to regeneration of *Maesa* or *Medicago* hairy roots after freezing in liquid nitrogen (results not shown).

6.3.2 Encapsulation-dehydration of hairy roots from Maesa lanceolata and Medicago truncatula

The application of the encapsulation-dehydration protocol is considered to be more complicated than vitrification and requires additional testing before it can be implemented for a many species. For encapsulation-dehydration two different protocols were tested. The first method was tested only for *Maesa lanceolata* hairy roots; the second one was applied for both *Maesa lanceolata* and *Medicago truncatula* hairy roots. The first protocol is based on a method for cryopreservation of *Vinca minor* hairy root cultures (Hirata et al. 2002). Root tips were precultured after isolation on 0.3M sucrose for three days and then encapsulated and dehydrated. The second protocol is based on a method for encapsulation – dehydration of apical meristem tips of *Melia azedarach* (Scocchi et al. 2004). Root tips were first encapsulated and then precultured on medium with an increasing sucrose gradient.

Results using protocol 1

Dehydration to very low intracellular moisture levels, so that crystallisation cannot occur, is essential for the survival of cryopreserved hairy roots. An experiment was therefore set up to determine the maximal dehydration time for encapsulated *Maesa* hairy roots. Hairy roots were dehydrated for up to 5 hours. Concurrently, they were weighed every hour and the percentage of the initial bead weight was determined (Fig 6-3a).

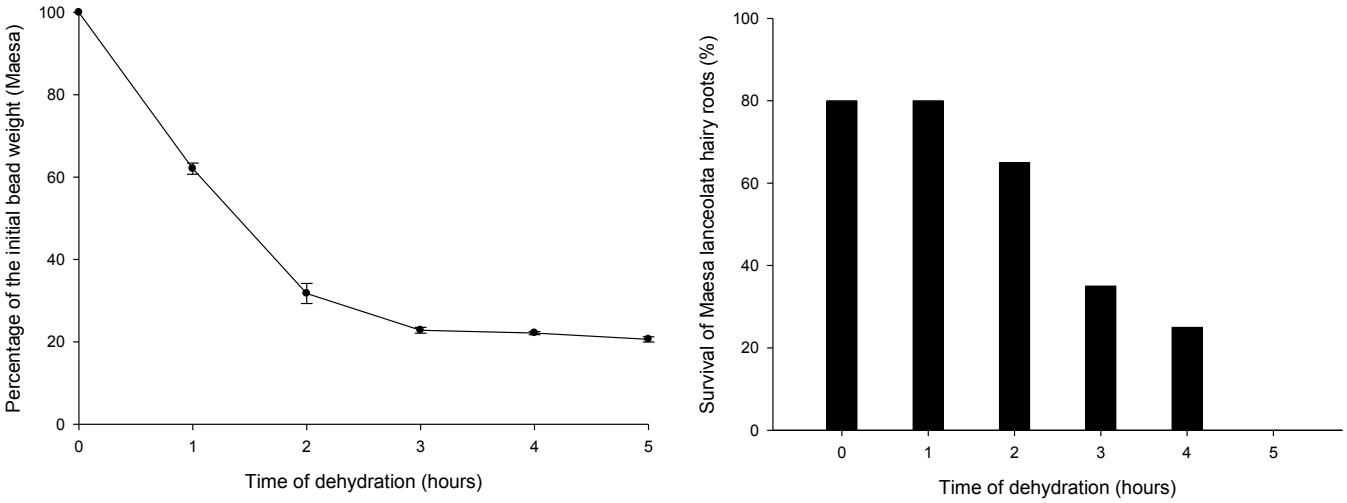


Fig 6-3 Control experiments to determine the optimal dehydration time for *Maesa lanceolata* hairy roots, using protocol 1 (without freezing). (Curve **a**) Encapsulated hairy roots of *Maesa* were dehydrated for 5 hours by the air of the laminar flow cabinet. Beads were weighed every 30 min. (Histogram **b**) Survival of the dehydrated hairy roots. Beads were placed on a plate with solid medium and survival rates were recorded 6 weeks after thawing. These were preliminary experiments that were not done in replicates; the experiment consisted of at least 10 hairy roots.

The weight of the beads decreased very fast until 2 – 3 hours of dehydration. From then on the weight of the beads was more or less constant, with a minimum of 20% after 5 hours of dehydration. Every hour during dehydration, control beads were placed on solid medium to regenerate. After 6 weeks the survival of the hairy roots was evaluated (Fig 6-3b). As expected, the survival of the dehydrated controls decreased with increasing dehydration time. The survival before dehydration was 80%, indicating that the preculture and encapsulation already had some negative influence on the viability of the hairy roots. After 1 hour of dehydration, the survival percentage was still 80%, however, from then on there was a continuous decline with no survival after 5 hours of dehydration. For further freezing experiments, samples were desiccated for 2 to 3 hours.

Survival percentages of the hairy roots after cryopreservation were very low (Fig 6-4).

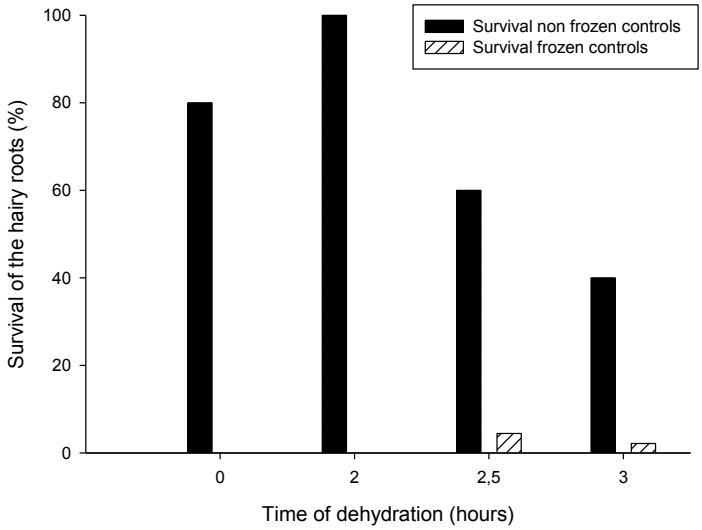


Fig 6-4 Survival rates of *M. lanceolata* hairy roots after cryopreservation. Hairy root beads were dehydrated for different time points before freezing. Survival rates were recorded 6 weeks after thawing. These were preliminary experiments that were not done in replicates; the experiment consisted of at least 10 hairy roots.

Viable hairy roots were only found when the samples were dehydrated for 2.5 or 3 hours, 4% and 2% respectively (Fig 6-4). Because of the low survival percentages in the initial experiments, this protocol was not further tested for *Medicago truncatula* hairy roots. Instead of optimizing this protocol we decided to try another protocol for encapsulation-dehydration of hairy root cultures.

Results using protocol 2

The maximal dehydration time was also determined using the second protocol. The weight loss of the beads was similar for *Maesa* and *Medicago* hairy root beads (Fig 6-5a).

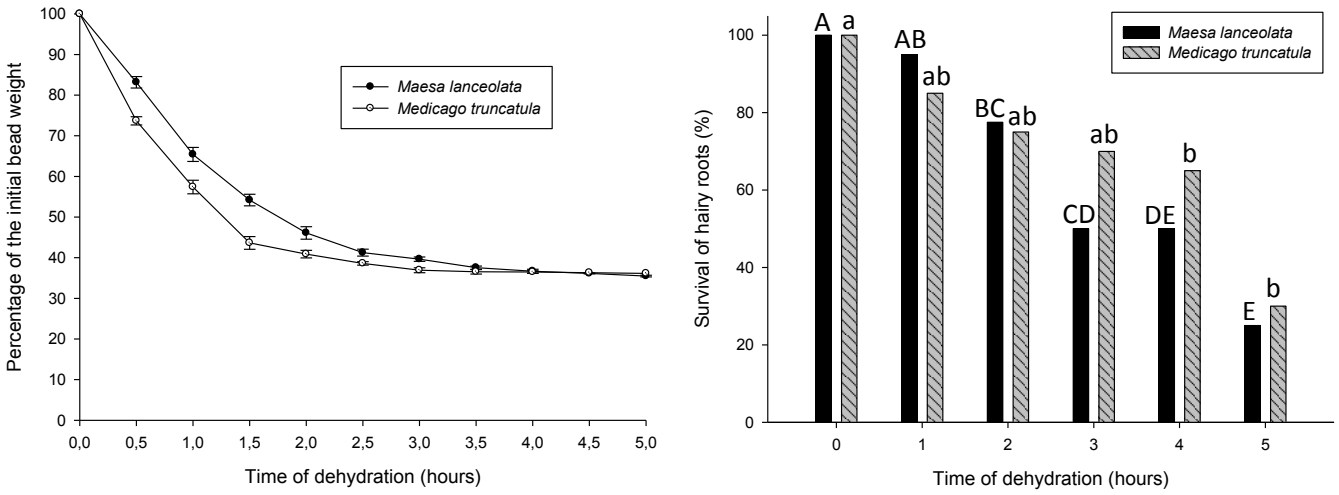


Fig 6-5 Control experiments to determine the optimal dehydration time for *Maesa lanceolata* and *Medicago truncatula* (without freezing). (Curve **a**) Encapsulated hairy roots of *Maesa* and *Medicago* were dehydrated for 5 hours by the air of the laminar flow cabinet. Beads were weighed every 30 minutes. (Histogram **b**) Survival of the dehydrated hairy roots of *Maesa* and *Medicago*. Beads were put on a plate with solid medium and survival rates were recorded 6 weeks after thawing.
Each experiment consisted of three repeats with at least 10 hairy roots in each repeat. Different letters indicate significant differences (P< 0.05), * = significant difference (P< 0.05) between two different treatments on the same time point; according to non parametric ANOVA.

The weight of the beads decreased rapidly until 2 hours of dehydration. From then on the weight of the beads was approximately constant, reaching a minimum after 5 hours of dehydration when 35% of the initial bead weight was achieved for *Maesa* and 36% for *Medicago*. Every hour during dehydration, control beads were placed on solid medium to regenerate. After 6 weeks the survival of the hairy roots was evaluated (Fig 6-5b). There were no significant different effects for *Maesa* and *Medicago*. The survival before dehydration was 100% for both species (Fig 6-5b, time point 0) and was 25% and 30% after 5 hours of dehydration for *Maesa* and *Medicago*, respectively. For both *Maesa* and *Medicago,* there is a continuous decline in survival percentages with no significant drops (Fig 6-5b).

Freezing of 0 to 4 hours dehydrated *Maesa lanceolata* hairy root tips was tolerated (Fig 6-6). Survival of 90% of the hairy roots was observed after 2.5 hours of dehydration, which represents no significant difference to the unfrozen controls. After 4 hours of dehydration, survival rate dropped to 20% after freezing. Because of these positive results, no further optimalization of the protocol was attempted for *Maesa lanceolata* hairy root conservation.

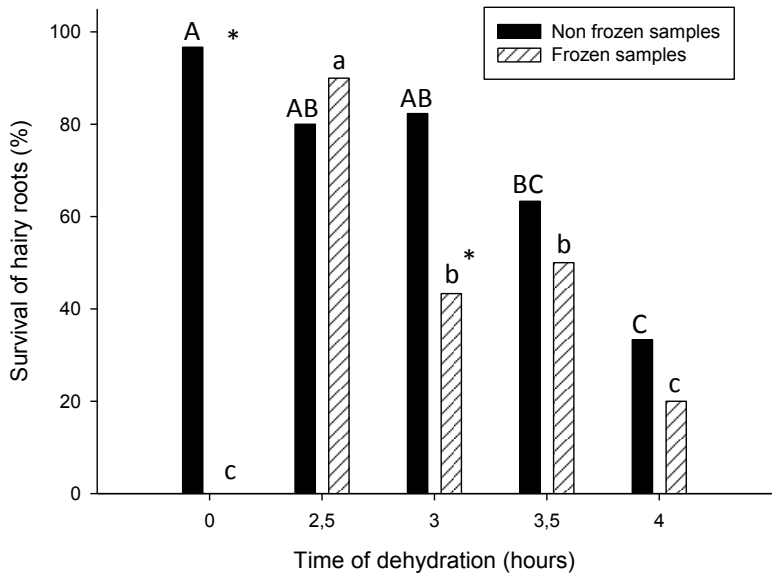


Fig 6-6 Survival rates of *Maesa lanceolata* hairy roots after cryopreservation. Hairy root beads were dehydrated for different time pints before freezing. Survival rates were recorded 6 weeks after thawing.
Each experiment consisted of three repeats with at least 10 hairy roots in each repeat. Different letters indicate significant differences ($P < 0.05$), '*' significant differences ($P < 0.05$) between two different treatments on the same time pint; according to non parametric ANOVA.

The survival after encapsulation-dehydration of *Medicago truncatula* was much lower compared to *Maesa lanceolata* (Table 6-1). The highest survival rate (16%) was obtained with samples dehydrated for 5 hours. However, during the three days of preculture, already 24% of the hairy root tips had grown out of the bead. This meant that about 1/4 of all hairy roots were not protected anymore against freezing damage. To prevent this outgrowth, we included the growth inhibitors abscisic acid (ABA) and paclobutrazol. These substances were added solely to the preculture media and not to the alginate or calcium solutions, to avoid negative effects after thawing. Using ABA, still 19% of the hairy roots had grown out of the bead after the preculture. Addition of paclobutrazol lowered this percentage to 7%. The survival rates of the hairy roots after freezing are given Table 6-1.

Table 6-1 Comparison of the effect of different growth inhibitors during preculture on the survival of *Medicago truncatula* hairy root tips. Hairy root beads were dehydrated for different time points and survival rates were recorded 6 weeks after thawing.

Growth inhibitor	Freezing	Dehydration Time	Survival (% ± SE)	
None	No	0	92.7 ± 3.7	a
	No	2	60.0 ± 15.3	ab
	No	3	60.0 ± 10.0	ab
	No	4	26.7 ± 3.3	b
	No	5	34.8 ± 10.6	b
	Yes	0	0.0	a
	Yes	2	3.3 ± 3.3	a
	Yes	3	10.0	a
	Yes	4	13.3 ± 3.3	a
	Yes	5	16.7 ± 12.0	a
ABA	No	0	96.7 ± 5.8	a
	No	2	80.0 ± 11.5	ab
	No	3	55.0 ± 5.0	b
	No	4	53.3 ± 8.8	b
	No	5	66.7 ± 8.8	ab
	Yes	0	0.0	c
	Yes	2	30.0	a
	Yes	3	30.0 ± 10.0	a
	Yes	4	25.0 ± 5.0	ab
	Yes	5	10.0 ± 5.8	bc
Paclobutrazol	No	0	83.3 ± 3.3	ab
	No	2	96.7 ± 3.3	a
	No	3	83.3 ± 3.3	ab
	No	4	80.0	b
	No	5	73.3 ± 3.3	b
	Yes	0	0.0	b
	Yes	2	0.0	b
	Yes	3	0.0	b
	Yes	4	36.7 ± 8.8	a
	Yes	5	53.3 ± 8.8	a

Each experiment consisted of three repeats with at least 10 hairy roots in each repeat. Different letters indicate significant differences ($P < 0.05$) within one treatment, according to non parametric ANOVA.

The highest survival rate using ABA as a growth inhibitor was 30%, while the highest survival rate using paclobutrazol as a growth inhibitor was 55%. Both ABA and paclobutrazol clearly inhibit growth of the hairy roots during preculture and don't have a negative effect on the viability of the hairy roots which results in higher survival percentages of hairy roots after freezing. Survival percentages were, however,

higher than expected so ABA and paclobutrazol have possibly other effects on the hairy roots, which make them more tolerant for dehydration and freezing.

For *Maesa lanceolata* as for *Medicago truncatula* we observed no morphological abnormalities after cryopreservation of the hairy roots (fig 1 e-h). The growth rates were also the same for non frozen and frozen samples (results not shown).

6.4 Discussion

To evaluate and change the production of pharmaceutical important saponins, hairy root lines were established from *Maesa lanceolata* and *Medicago truncatula*. Using a standard protocol for *Agrobacterium* mediated transformation we obtained hairy root lines from both species. In order to maintain the original cultures we have developed a cryopreservation protocol.

Because vitrification involves less handling steps in comparison to other cryopreservation techniques (Engelmann 2004), we first tried this method for the cryopreservation of *Maesa lanceolata* and *Medicago truncatula* hairy roots. However, the PVS2 solution commonly used for vitrification lead to significant damage of the hairy root tips, also observed for some other tissue and cell structures (Volk et al. 2006). Only very low percentages of the *Maesa lanceolata* and *Medicago truncatula* hairy roots survived incubation of 5 minutes and longer in PVS2, without pretreatment with a loading solution. Roots that were first treated with loading solution seemed to be more resistant to PVS2 solution, as survival percentages decrease from 10 – 15 minutes of treatment with PVS2. However, none of the *Maesa* or *Medicago* hairy roots survived freezing in liquid nitrogen after vitrification. In literature, there are only three studies in which vitrification is successfully applied on hairy roots. There is a vitrification procedure described for *Panax ginseng* (Yoshimatsu et al. 1996)*, Angelica acutiloba* (Yoshimatsu 2000) and *Atropa belladonna* (Touno et al. 2006) hairy roots. Survival percentages are respectively, 60%, 96% and 93%. On the other hand, there are also reports that confirm the negative effect of PVS2 solution on the viability of plant material of some species (Volk et al. 2006; Xue et al. 2008). Xue *et al.* (2008) tried to cryopreserve hairy roots of three different species using encapsulation-vitrification. For this technique hairy roots were also encapsulated in calcium-alginate beads but were desiccated through treatment with PVS2 solution (Sakai 2000; Xue et al. 2008). No hairy roots of *Gentiana macrophylla* and *Astragalus membranaceus* survived when using the PVS2 solution to vitrify the material. Only 25% of the hairy roots of *Eruca sativa* survived treatment with PVS2 solution (Xue et al. 2008). In view of our own negative

results and the few positive results reported in literature on cryopreservation of hairy roots, we concluded that vitrification was not a suitable method to preserve hairy root cultures.

Encapsulation-dehydration is a cryopreservation method that is more labour intensive than vitrification but does not require treatment with cytotoxic compounds (Niino and Sakai 1992). Hairy roots are precultured in medium with high sucrose concentrations (before or after encapsulation), encapsulated in calcium-alginate beads and then dehydrated by the air of a laminar flow cabinet. We tested two different protocols for encapsulation – dehydration of hairy roots. The first method was based on a protocol for *Vinca minor* hairy roots (Hirata et al. 2002). However, initial tests with *Maesa lanceolata* hairy roots showed that this protocol was also not suitable for cryopreservation. Highest survival percentage after freezing was only 4%. Much better results were obtained with the second protocol, based on encapsulation – dehydration of *Melia azedarach* apical meristem tips (Scocchi et al. 2004). For *Maesa lanceolata* hairy roots we obtained survival percentages of 90% after encapsulation-dehydration. For *Medicago truncatula* we first encountered the problem that the hairy roots grew too fast and grew out of the bead even before freezing. In this way the root tips were not protected anymore against freezing damage and this resulted in very low percentages of survival. Isolation of smaller root tips was technically too difficult for practical use. So to overcome this problem we added growth inhibitors to the preculture medium, namely ABA and paclobutrazol. Both substances are frequently used as growth retardants in cryopreservation and cold preservation of plant material (Cha-Um et al. 2007; Hirata et al. 1998; Hirata et al. 2002). ABA is also generally known as a plant hormone that plays a role in the acquisition of desiccation tolerance and osmotic stress (Ingram and Bartels 1996; Sharp et al. 2004). ABA induces late-embryogenesis-abundant (*lea*) genes, which encode LEA proteins. These proteins have increased levels during drought and are involved in dehydration tolerance in plants (Ingram and Bartels 1996). Paclobutrazol is a growth retardant that has a negative effect on root growth but it is also an inhibitor of ABA catabolism, so addition of paclobutrazol may increase the endogenous ABA levels in *Medicago truncatula* hairy roots during desiccation and as a consequence *lea* genes may be induced which might make the hairy roots less susceptible to drought stress (Cha-Um et al. 2007; Krizan et al. 2006). Addition of ABA or paclobutrazol to the preculture media resulted in survival percentages of respectively 30% and 53%. We believe that these higher percentages are partly due to the reduction of outgrowth and partly due to the higher desiccation tolerance, upon addition of plant growth regulators ABA and paclobutrazol.

There are very few reports on encapsulation-dehydration of hairy roots. The first one was published by Hirata *et al.* (1998), describing an encapsulation-dehydration protocol for Horseradish hairy root

cultures. They obtained survival rates of 60% of the cryopreserved hairy roots. Later, the same group published a report on encapsulation-dehydration of *Vinca minor* hairy roots (Hirata et al. 2002). A survival rate of more than 70% was obtained in this case. Our results show that for *Maesa lanceolata* 90% and for *Medicago truncatula* 53% of the hairy roots can be regenerated after cryopreservation. The same protocol is potentially useful for other species, with ABA and paclobutrazol as possible cryoprotectants. In the future we will use the established encapsulation-dehydration protocol routinely for the storage of large numbers of different transgenic root lines of *Maesa lanceolata* and *Medicago truncatula*.

6.5 Materials and methods

6.5.1 Plant material

Maesa lanceolata seeds were collected in Moshi, Tanzania by Frank Mbago (Department of Botany, University of Dar-Es-Salaam). The method for sterilization and *in vitro* cultivation is described in the Materials and methods section of Chapter 2.
Medicago truncatula plant material of the cultivar Jemalong J5 was obtained from the group of Marcelle Holsters (VIB – department of Plant Systems Biology – Research group on plant microbes). Seeds of the plants were harvested and scarred with concentrated sulphuric acid. Sterilization was performed using 12% (w/v) sodium hypochlorite. After a 3 to 4 hours treatment with 1mM 6-benzylaminopurine (BAP), germination of the seeds took place on wet and aseptic Whatmann paper in the dark at 25°C.

6.5.2 Production and maintenance of hairy root cultures

The method for hairy root induction of *Maesa lanceolata* is described in the Materials and methods section of Chapter 5.
Medicago truncatula hairy roots were induced using *Agrobacterium rhizogenes* (strain LBA 9402/12) transformation on leaf discs or seedlings. The *Agrobacterium* strain was transformed with the pK7WG2D plasmid, in which an eGFP-ER gene was inserted after a 35S promoter sequence. *Agrobacteria* were transferred from a glycerol stock to 3ml of liquid YEB medium. This medium contained antibiotics (100mg/l rifampicin, 100mg/l spectinomycin and 300mg/l streptomycin) and consisted of 5g/l beef extract, 1g/l yeast extract, 5g/l peptone, 0.15M sucrose and 2ml/l 1M $MgSO_2$. The bacterial cultures were incubated on a rotary shaker (220 rpm) at 28°C. After 48 hours 20µl of this bacterial preculture is brought into 5ml of fresh YEB medium with the same antibiotic concentrations as mentioned before. The bacterial cultures were again incubated on a rotary shaker (220 rpm) at 28°C. 48 hours later, these cultures can be used for the transformation.
Medicago truncatula hairy roots were induced by wounding seedlings. Wounded seedlings were dipped into the bacterial culture and afterwards placed on solid MS basal medium supplemented with 0.8% (w/v) agar, 0.03M sucrose and 1g/l BA. After 7-10 days of co-cultivation with the *Agrobacteria*, the leaves were placed on solid MS basal medium supplemented with 0.8% (w/v) agar, 0.03M sucrose and 500 mg/L cefotaxime. Hairy roots were isolated from the seedlings after 15-30 days and were placed on MS basal medium supplemented with 0.8% (w/v) agar and 0.03M sucrose.
Both hairy root cultures were cultured in the dark at 25°C and were subcultured every month. For the selection of the transgenic material, GFP was used as a visible marker. Hairy roots that showed green fluorescence were considered as transgenic.

6.5.3 Vitrification

To test the toxicity of the plant vitrification solution 2 (PVS2: 0.4M glycerol, 15% (v/v) DMSO, 15% (v/v) ethylene glycol and 0.4M sucrose) (Sakai et al. 1990), *Maesa lanceolata* and *Medicago truncatula* hairy root tips (+/- 5 mm in length) were excised from 2-week old root cultures and precultured for 3 days at 25°C in the dark on SH solid medium (*Maesa*) or MS solid medium (*Medicago*) containing 0.3M sucrose. After the preculture, the samples were transferred to cryovials (10 root tips/cryotube) and incubated at 0°C in 2ml loading solution (2M glycerol and 0.4M sucrose) for 10 minutes. The loading solution was removed and 2ml cooled PVS2 solution (0°C) was added to the root tips. Different incubation times (0, 2, 5, 10, 15 and 20 minutes) with PVS2 solution were tested. After PVS2 incubation, samples were plunged into liquid nitrogen. After storage for 3 days, samples were thawed rapidly in a water bath at 40°C and washed for 5 minutes with 2ml of basal medium containing 1M sucrose. Afterwards the hairy roots were placed on SH or MS solid medium with 0.09M sucrose.
To assess the toxicity of PVS2, root tips were treated the same way as described above but after treatment with PVS2 solution, the root tips were directly washed for 5 minutes with basal medium containing 1M sucrose and were placed on SH or MS solid medium with 0.09M sucrose. To determine the effect of loading solution on the survival of hairy roots after PVS2 treatment, half of the samples were pretreated with loading solution for 10 min and the other half of the samples were directly incubated in PVS2 solution. Different incubation times with PVS2 were tested (0, 2, 5, 10, 15 and 20 minutes).
Trypan blue staining was performed for some samples to get an idea about the viability of the hairy roots directly after thawing. For this staining, root tips were put in a drop of trypan blue (Fluka) on a microscopic slide.
However, in all cases the survival rate was recorded as the percentage of hairy roots showing definite elongation or side root formation after cultivation for 6 weeks in the dark at 25°C.

6.5.4 Encapsulation-dehydration

Protocol 1
Root tips (2-3 mm in length) were excised from 2 week old hairy root cultures of *Maesa lanceolata*. The isolated root tips were placed on SH medium including 0.3M sucrose and 2µM ABA. After a 3-days preculture, the root tips were suspended in 100ml of 2% (w/v) sodium alginate solution containing 0.3M sucrose and 2µM ABA. Then the suspension is dropped into 100ml 50mM $CaCl_2$ with 0.3M sucrose. Solidification takes about 15 minutes and afterwards the beads were transferred to a Petri dish and dehydrated by placing the dish in the air current of a laminar flow hood for 0 to 3 hours, until the bead weight attained +/- 25% of the initial weight. Dehydrated beads were placed in cryovials (10 beads/cryovials) and immediately immersed in liquid nitrogen.
After storage of 3 days, the beads were thawed rapidly in a water bath at 40°c and transferred without washing onto solid SH medium containing 0.09M sucrose. The survival rate was recorded as the percentage of hairy roots growing out of the beads after cultivation for 6 weeks in the dark at 25°C.

Protocol 2

Root tips (2-3 mm in length) were excised from 2 week old root cultures of *Maesa lanceolata* and *Medicago truncatula*. The isolated root tips were immediately suspended in 100ml of 3% (w/v) sodium alginate solution containing 0.1M sucrose and dropped into 100ml 0.1M $CaCl_2$, plus 0.1M sucrose. The roots were kept in the solution for 10 min to allow solidification of the calcium alginate beads. Next, the beads were transferred to a shake flask containing 25ml liquid SH medium for *Maesa*, or MS medium for *Medicago*, supplemented with 0.5M sucrose (50 beads/shake flask). After 24h, the medium was replaced with medium containing 0.75M sucrose and 24 hours later with medium containing 1M sucrose. Continued growth of *Medicago truncatula* caused extrusion of the root tips from the alginate beads that render them unsuitable for freezing. Outgrowth of *Medicago truncatula* root tips was reduced by adding 3mM ABA (abscisic acid) or 0.01mM paclobutrazol (Bonzi®) to the preculture media. Optimal concentrations of ABA and paclobutrazol for growth reduction of *Medicago truncatula* hairy roots were determined in previous experiments (unpublished results).

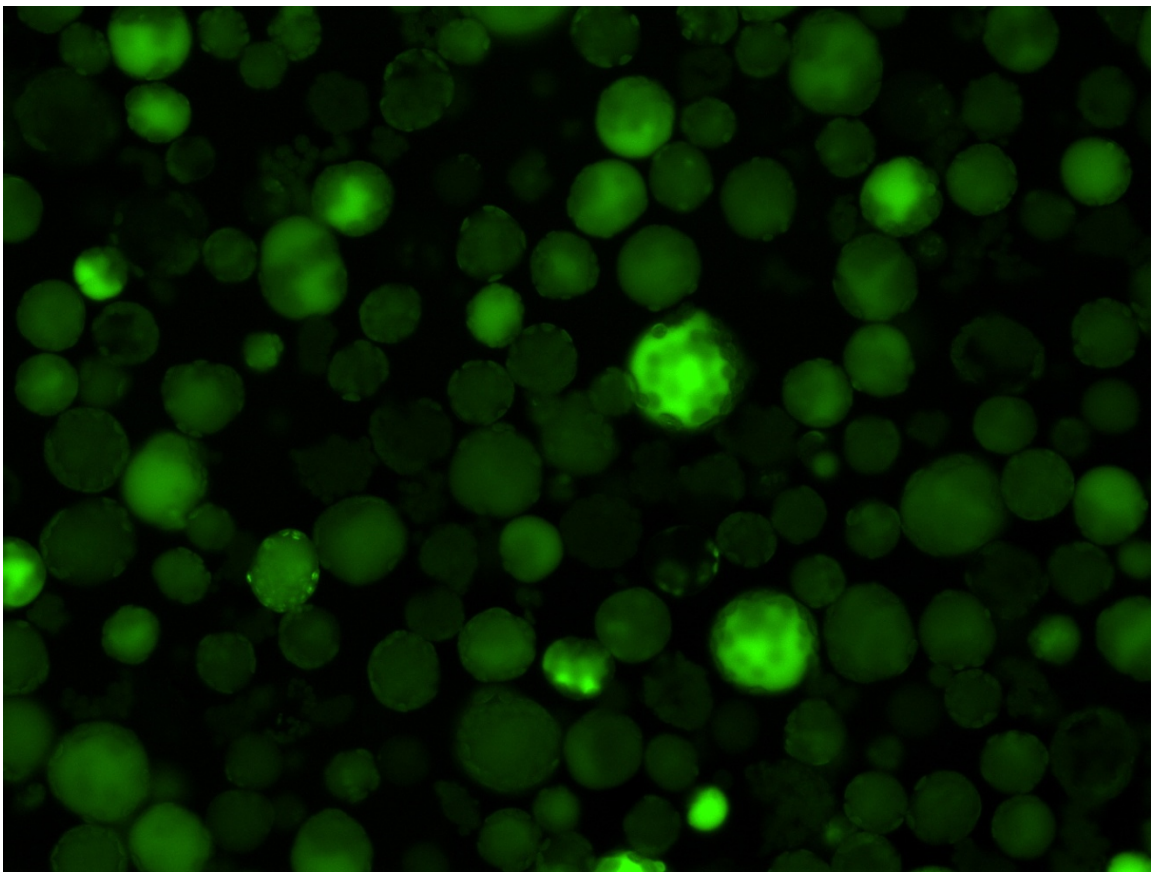
After three days of preculture in medium with increasing sucrose concentrations, the encapsulated root tips were transferred to a Petri dish and dehydrated by placing the dish in the air current of a laminar flow hood for 0 to 5 hours, until the bead weight attained +/- 35-40 % of the initial weight. Dehydrated beads were placed in cryovials (30 beads/cryovials) and then directly immersed in liquid nitrogen.

After storage for 3 days, the beads were thawed rapidly in a water bath at 40°C and then transferred without washing onto solid SH medium containing 0.09M sucrose (*Maesa*) or onto solid MS medium containing 0.03M sucrose (*Medicago*). The survival rate was recorded as the percentage of hairy roots growing out of the beads after cultivation for 6 weeks in the dark at 25°C.

6.5.5 Statistical analysis

Experiments were done in triplicate, each replicate containing a minimum of ten samples. The data represent mean values from the three independent replicates. Data were nonnormal, hence, ranking was calculated using proc rank (SAS 9_1_3 software). Statistical differences between samples were determined by non parametric oneway analysis of variance, also using SAS 9_1_3 software. Graphs were established using SigmaPlot 10 software.

CHAPTER 7 MAESA PROTOPLAST RESEARCH



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7.1 Abstract

Somatic hybridization through protoplast fusion has been shown to create diversity in secondary metabolites. In this chapter, an efficient protocol for protoplast isolation was established for *Maesa argentea, M. balansae, M. lanceolata* and *M. perlarius* calli, *in vitro* shoots and hairy roots. A general enzyme mixture comprising 1.5% cellulase, 0.5% macerozyme/pectinase and 0.5M mannitol proved efficient for the different types of explants and species. Protoplast yields when using callus material were 7 x 10^4 protoplasts $gram^{-1}$ fresh weight for *M. argentea* and up to 1 x 10^6 protoplasts $gram^{-1}$ fresh weight for *M. perlarius.* Maximum protoplast numbers for leaf material were between 2 to 7 x 10^6 protoplasts $gram^{-1}$ fresh weight and much less variation was noticed between different species compared to callus material. For efficient protoplast isolation, *Maesa lanceolata* hairy roots were precultured first on medium containing indole-3-butyric acid to induce higher number of lateral roots. Yields for hairy roots as explant material were around 9 x 10^6 protoplasts $gram^{-1}$ fresh weight. A technique for automatic counting of protoplasts was also established using a Coulter Counter Multisizer™ device. In addition, a substantial effort was made to find conditions that could promote the regeneration of protoplasts. Cultured protoplasts showed sustained viability but only few protoplasts formed a new cell wall. The limited number of walled cells was insufficient to allow regeneration to take place. Finally, preliminary tests were performed to adapt a protocol for transient transformation of *Arabidopsis thaliana* mesophyll protoplasts for transformation of *Maesa lanceolata* protoplasts. Although transgenic cells were observed, the transformation frequency was far too low for practical testing of metabolite genes. Further optimization of the conditions is required.

7.2 Introduction

Protoplasts are 'naked' cells of which the cell wall has been removed mechanically or enzymatically. Already at the beginning of the sixties, E.C. Cocking used an enzyme preparation for the degradation of protoplasts and yielded single protoplasts from tomato root tips (Cocking 1960). Nevertheless, it took until 1968 before Takebe and co-workers developed a technique for the production of large amounts of active protoplasts from mesophyll cells of *Nicotiana tabacum* (Takebe et al. 1968).

Isolated protoplasts are exploited in many studies involving membrane function, cell structure and cell physiology (Binder et al. 2003; Steffens et al. 2001; Swanson et al. 1998) but are also used for toxicological assessments (Kristen 1997) and the study of plant-virus interactions (Vishnichenko and Zavriev 2001). In addition, uptake of isolated DNA into protoplasts provides the basis for transient and stable nuclear transformation. Furthermore, somatic hybridization through protoplast fusion allows (full or partial) combination of nuclear and cytoplasmic genomes at interspecific and intergeneric levels. This technique is often used for circumventing naturally occurring sexual crossing barriers (Davey et al. 2005).

During the last decades, the isolation, fusion and culture of protoplasts has been described for a diverse range of plant species (Davey et al. 2005), including the saponin producing alfalfa (*Medicago sativa*) (Jin et al. 2003), common bulb onion (*Alium cepa*) (Karim and Adachi 1997), camphor tree (*Cinnamomum camphora*) (Du and Bao 2005), date palm (*Phoenix dactylifera*) (Chabane et al. 2007) and red cabbage (*Brassica oleracea*) (Chen et al. 2004). A few studies report a change in secondary metabolism after somatic hybridisation. One example is the formation of novel glycoalkaloids in somatic hybrids between cultivated and wild potato species. Fusion of protoplasts of *Solanum brevidens* and *Solanum tuberosum* led to production of secondary metabolites that were not found in both parents (Laurila et al. 1996) (Fig 7-1). *S. tuberosum* produces solanidine and *S. brevidens* contains tomatidine, both glycoalkaloids are derived from the precursor teinemine. Fusion of leaf protoplasts from both parents led to the establishment of somatic hybrids. Chromatographic analysis showed that all hybrids produced demissidine, a component that was not found in the parental species.

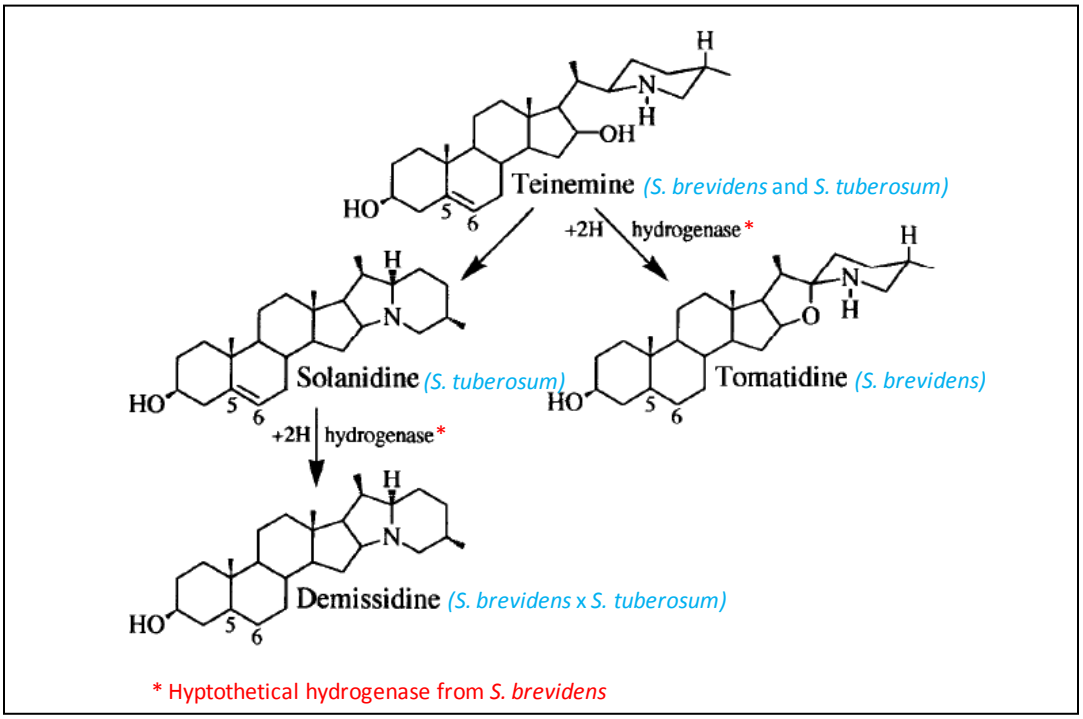

Fig 7-1 A hypothetical pathway for the production of demissidine in somatic hybrids between *Solanum brevidens* and *S. tuberosum*. Figure adapted from Laurila et al. (1996).

This could be explained by hypothesizing a hydrogenase encoded by *S. brevidens.* The common precursor, teinemine has a double bond in its structure. Solanidine too has this double bond but tomatidine and demissidine do not. The hypothetical hydrogenase could mediate the production of

tomatidine in *S. brevidens* through hydrogenation of the double bond in teinemine. In somatic hybrids, the hydrogenase would also be able to hydrogenate the double bond of solanidine, leading to the production of demissidine (Laurila et al. 1996). A similar phenomenon was observed by Saverese and co-workers when analysing the glycoalkaloid content of somatic hybrids of *Solanum tuberosum* and *Solanum bulbocastanum* (Savarese et al. 2009). In a last example, researchers were able to make asymmetric hybrids through protoplast fusion between *Panax ginseng* and *Daucus carota*. The hybrid calli contained different concentrations of the most important ginseng saponin (ginsenoside Rb1) and additionally showed other unidentified secondary metabolites in the HPLC chromatogram (Han et al. 2009).

For the majority of the protoplast applications, the protoplast yield is a critical factor and therefore needs to be accurately determined. Classically, protoplasts are counted manually using a haemocytometer or counting chamber, but this is a time-consuming step. Depending on the species, the physiological status of the plant and the type explant used, the half-life of protoplasts can range from a few hours to several days. In cases the half-life expectancy is short; the manual counting of protoplasts may become inadequate. We propose here an automatic method for counting of plant protoplast. The Coulter Counter Multisizer™ is a device that determines the size distribution of particles in solution within one measurement. The technique was originally developed for quick and accurate counting of blood cells. Nowadays it is used for a much wider field of applications, for example the study of sediments in erosion (Law et al. 2008), bacterial spores (Buhr et al. 2008), liposomal complexes (Klegerman et al. 2010), oil emulsions (Silset et al. 2010) and human cells, e.g. osteoblasts or adipose cells (McLaughlin et al. 2010; Slapnicka et al. 2008). For Multisizer™ measurements, particles are suspended in a weak electrolyte solution and drawn through a small aperture with two electrodes that have an electric current flowing between them. When the particles pass through the aperture, they displace their own volume that increases the impedance of the aperture. This change produces a pulse that is digitally processed in real time. The 'Coulter Principle' states that this pulse is directly proportional to the tri-dimensional volume of the particle that produced it. Analyzing these pulses enables a size distribution to be acquired and displayed in volume and diameter. In addition, a metering device is used to draw a known volume of the particle suspension through the aperture; a count of the number of pulses can then yield the concentration of particles in the samples.

The objectives of this study were to analyze protoplast yield starting from different types of *in vitro* cultures of four *Maesa* spp. and to obtain fluorescent protoplasts that would facilitate the monitoring of protoplast fusion and selection of fusion products. In addition we developed a method to count protoplasts automatically, using a Coulter Counter Multisizer^{TM}. Isolated protoplasts were tested for regeneration using different culture types and medium constitutions. Finally, we started preliminary testing for transient transformation of *Maesa lanceolata* protoplasts.

7.3 Results

7.3.1 High efficient protoplast isolation from different *Maesa in vitro* cultures

Callus

The first material tested for protoplast isolation was *Maesa lanceolata* callus. The callus was induced on leaf discs incubated on 2,4-D containing medium (Chapter 3). Small-scale preliminary tests (without repetitions) were performed to find conditions required for efficient protoplast isolation. The basic enzyme mixture used consisted of cellulase RS, macerozyme R-10, pectolyase Y-23 and mannitol. We varied cellulase, macerozyme, pectolyase and mannitol conditions. In addition we tested sorbitol as osmoprotectant and we tested if shaking during protoplast isolation was beneficial. Callus was incubated for 5 hours in the enzymatic solution to allow subsequent manipulation of the protoplasts within a single day. The results are shown in Figure 7-2.

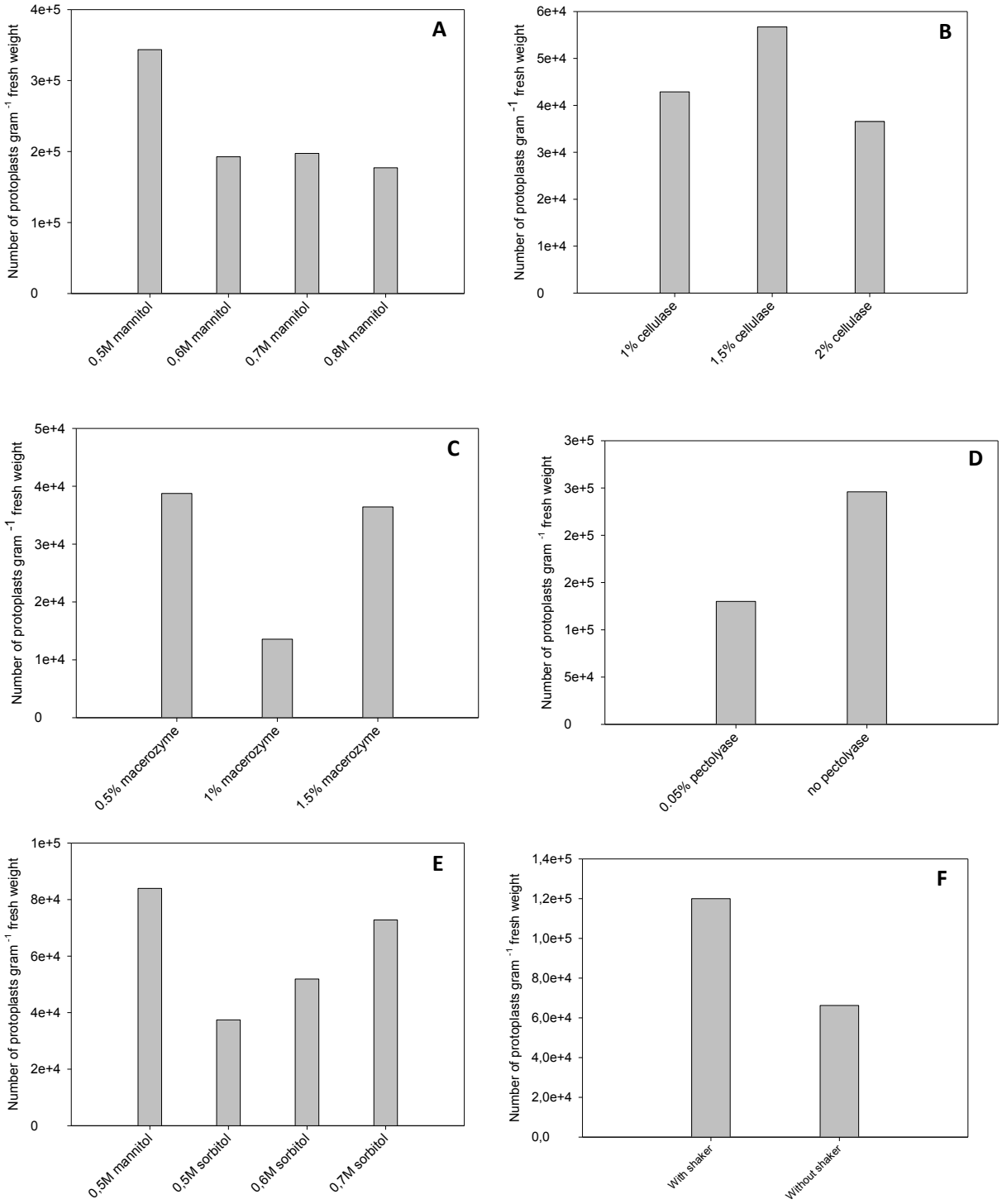

Fig 7-2 Small-scale preliminary tests to determine an efficient enzyme mixture for *M. lanceolata* callus. The basic mixture consisted of 2% cellulase RS, 0.5% macerozyme R-10 and 0.5M mannitol. In 6 independent tests we varied the cellulase concentration (histogram **a**), the macerozyme concentration (histogram **b**), the pectolyase concentration (histogram **c**) and the mannitol concentration (histogram **d**). We also tried sorbitol as an osmoprotectant in different concentrations (histogram **e**) and we tested if shaking during incubation in the enzyme mixture was necessary (histogram **f**). No repeats were included in the measurements.

Based on these preliminary tests, the enzyme mixture comprising 1.5% cellulase RS, 0.5% macerozyme R10 and 0.5M mannitol was used as the basic mixture for further experiments. Samples were put on a rotary shaker at 50 rpm during isolation. Using this protocol, an average of 2.7 x 10^5 protoplasts $gram^{-1}$ fresh weight was achieved for *M. lanceolata* callus material (Table 7-1).

Because of practical reasons we also tried to adjust the protocol in such a way that we could perform the protoplast isolation overnight instead of 5 hours. 1.5% cellulase R-10 was used instead of 1.5% cellulase RS and isolation took place during the night for 15 hours. The incubation conditions were not changed. This isolation protocol yielded 2 times more protoplasts, with a mean of 5.7 x 10^5 protoplasts $gram^{-1}$ fresh weight (Table 7-1).

Table 7-1 Comparison of different types of starting material for protoplast isolation from *Maesa lanceolata* and effect of incubation time on protoplast yield. Protoplast diameters are also represented. Results are mean values of three independent repeats.

		# protoplasts x 10^5 $gram^{-1}$ fresh weight ± SD	Mean diameter of the protoplasts
In vitro leaves	5 hours incubation	61.0 ± 1.90[a]	23 µm
	15 hours incubation	52.0 ± 140[a]	24 µm
Callus from leaves	5 hours incubation	2.7 ± 1.00[a]	30 µm
	15 hours incubation	5.7 ± 0.90[b]	30 µm
Callus from hairy roots	5 hours incubation	4.5 ± 0.90[a]	29 µm
	15 hours incubation	3.2 ± 0.06[b]	30 µm
Hairy roots	5 hours incubation	8.8 ± 2.50[a]	14 µm
	15 hours incubation	5.0 ± 1.80[a]	15 µm

Different letters indicate significant differences ($P < 0.05$) within one type of explant; according to independent T-test.

In subsequent experiments, callus from three other *Maesa* spp. was tested for protoplast isolation, using the same basic protocol but varying the composition of the enzyme mixture. Callus from *Maesa lanceolata* was also tested with all enzyme mixtures (Fig. 7-3).

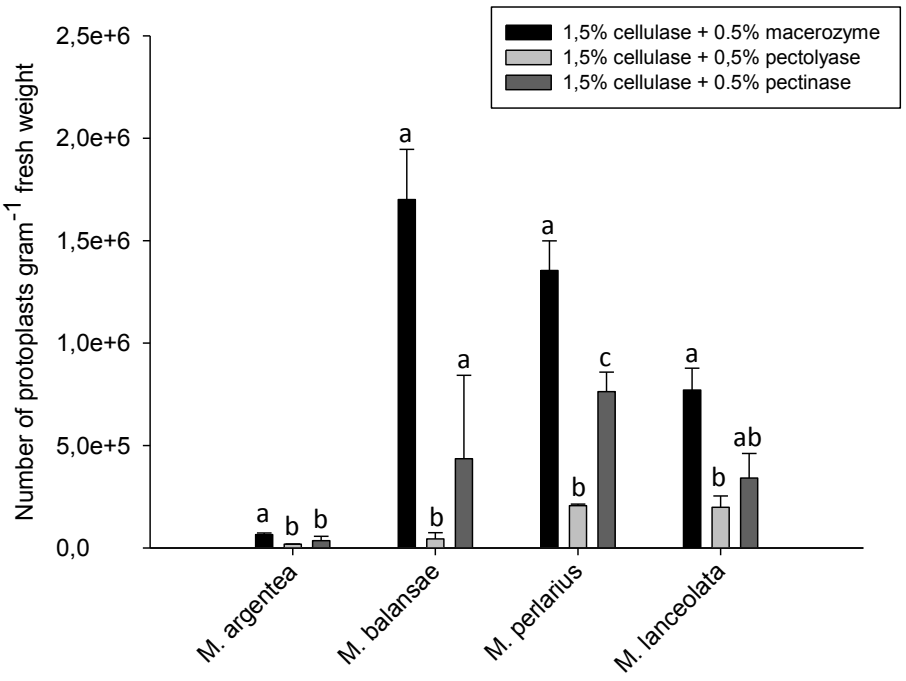

Fig 7-3 Callus of four different *Maesa* spp. was tested for protoplast isolation. 1.5% cellulase R10 was tested in combination with 0.5% macerozyme R10, pectolyase Y23 or pectinase.
Different letters indicate significant differences ($P < 0.05$) between different treatments within one species; according to Tukey test.

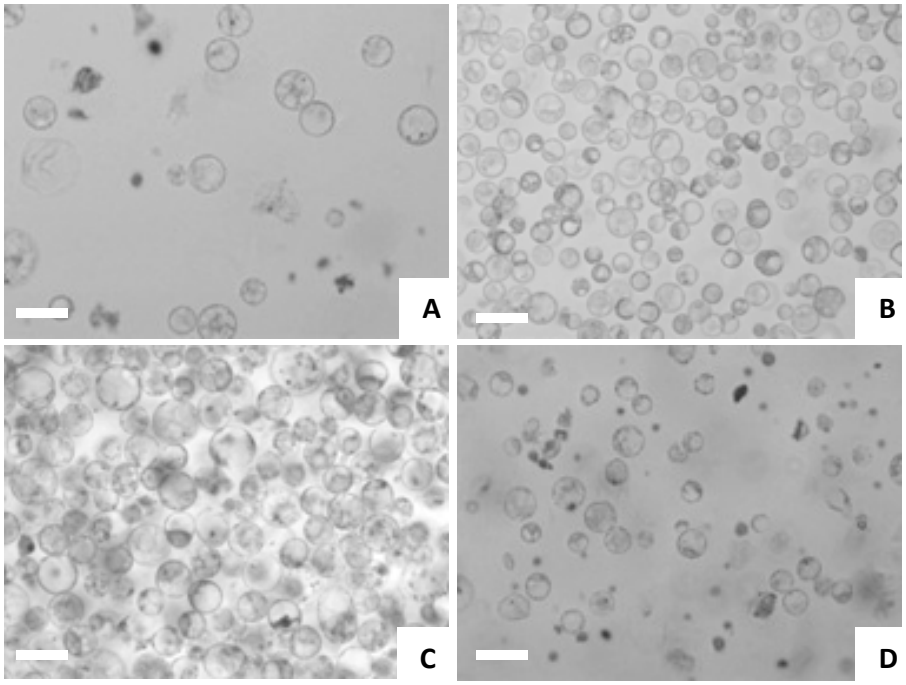

Fig 7-4 Protoplasts isolated from callus material (induced on leaves) of different *Maesa* spp. with the optimal enzyme mixture; 1.5% cellulase + 0.5% macerozyme for *M. argentea* (**a**), *M. balansae* (**b**), *M. lanceolata* (**c**) and *M. perlarius* (**d**). Scale bar = 50µm.

In general, the enzyme mixture consisting of 1.5% cellulase in combination with 0.5% macerozyme gave the best results, however, for *M. lanceolata* and *M. balansae* there was no significant difference with the enzyme mixture comprising 1.5% cellulase and 0.5% pectinase. Protoplast isolation was most efficient for *M. balansae* with approximately 1.7 x 10^6 protoplasts per gram fresh weight and *M. perlarius* with 1.4 x 10^6 protoplasts per gram fresh weight. *M. lanceolata* callus yielded 7.7 x 10^5 protoplasts per gram weight. For *M. argentea* we could not isolate large amounts of protoplasts, the maximum amount we achieved was 6.6 x 10^4 protoplasts per gram fresh weight. The enzyme mixture with macerozyme will be used in later experiments. We also tested the viability of the protoplast using trypan blue staining and this demonstrated that, for all species, viability was over 90% immediately after isolation. Protoplasts of the different species are represented in Figure 7-4.

In addition, it was possible to isolate protoplasts from callus that was induced on transgenic, GFP expressing *Maesa lanceolata* hairy roots. The number of protoplasts from callus as well as leaves was about 4.5 x10^5 protoplasts $gram^{-1}$ fresh weight (Table 7-1). The protoplasts from callus induced on hairy roots showed green fluorescence (Fig 7-5), which could be useful to distinguish fusion products in future experiments.

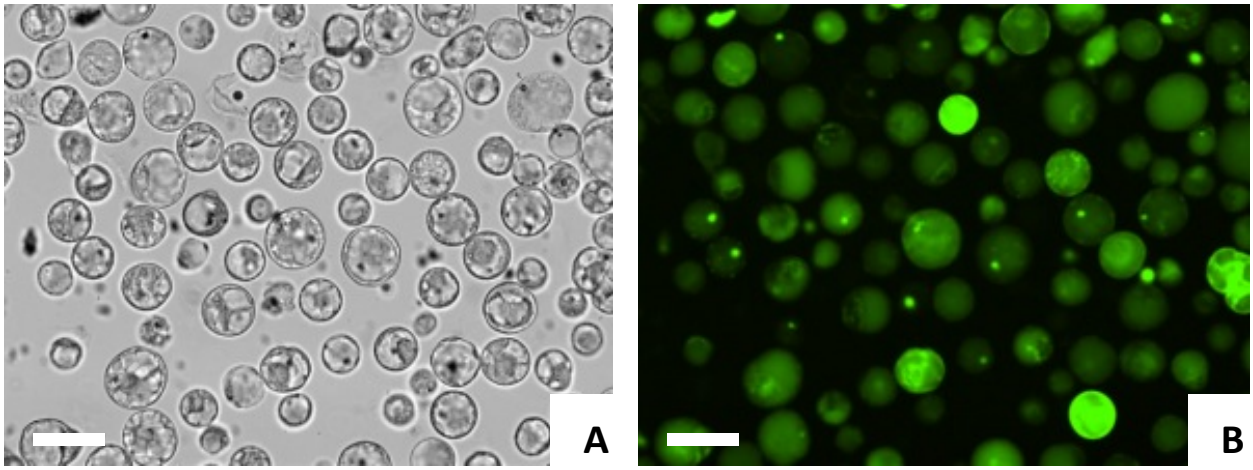

Fig 7-5 Protoplasts isolated from *M. lanceolata* callus induced on transgenic, GFP expressing hairy roots. The protoplasts show clear GFP fluorescence. Scale bar = 50µm.

Leaf material

Leaf material of *in vitro* plants from all four species was tested as explant material for protoplast isolation. We tested the same enzyme mixtures as for callus material (Fig 7-6). The isolation was performed overnight.

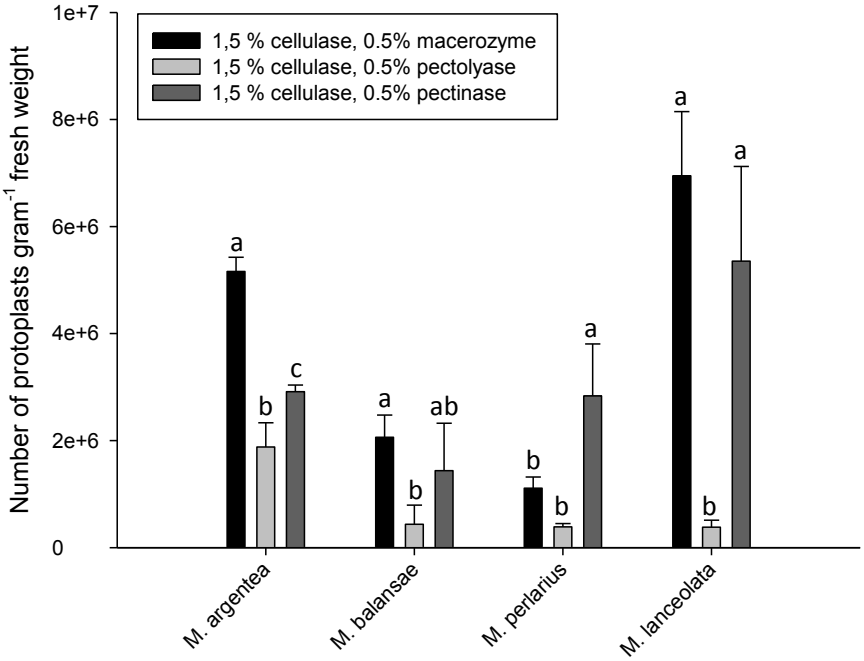

Fig 7-6 Leaf material of four different Maesa spp. was tested for protoplast isolation. 1.5% cellulase R10 was tested in combination with 0.5% macerozyme R10, pectolyase Y23 or pectinase.
Different letters indicate significant differences ($P < 0.05$) between different treatments within one species; according to Tukey test.

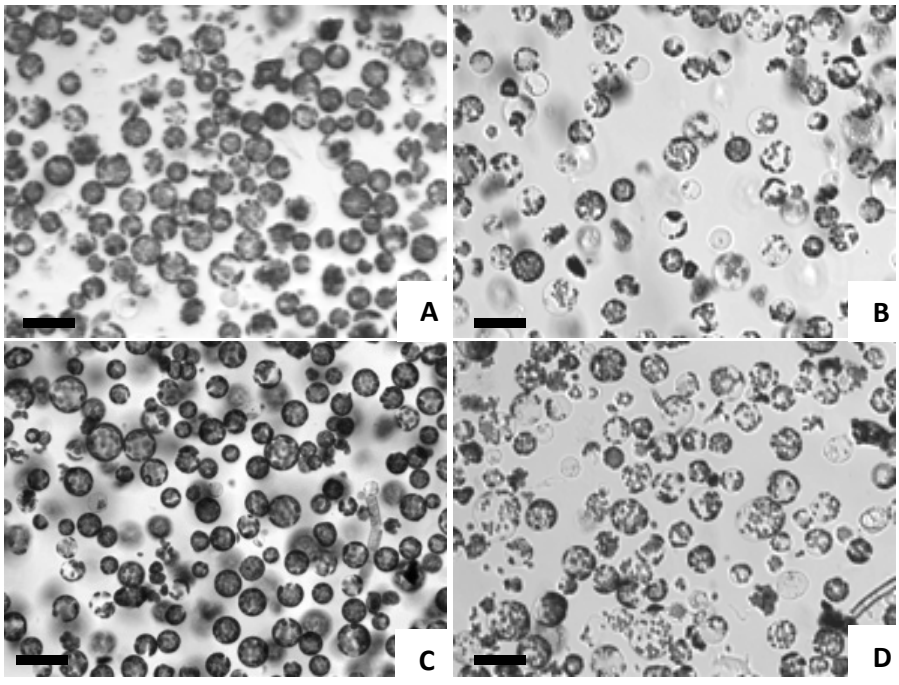

Fig 7-7 Protoplast isolated from leaf material of different *Maesa* spp. with the optimal enzyme mixture; 1.5% cellulase + 0.5% macerozyme for *M. argentea* (**a**), *M. balansae* (**b**) and *M. lanceolata* (**c**); 1.5% cellulase + 0.5% pectinase for *M. perlarius* (**d**). Scale bar = 50µm.

In general, the same enzyme mixtures were more efficient for protoplastation of leaf material compared to callus (Fig 7-6). Maximum protoplast yields were 6.9 x 10^6 $gram^{-1}$ fresh weight for *M. lanceolata*, 5.2 x 10^6 for *M. argentea*, 2.8 x 10^6 for *M. perlarius* and 2.1 x 10^6 for *M. balansae*. The enzyme mixture with macerozyme was clearly the best for *M. argentea*, while the enzyme mixture with pectinase was better for *M. perlarius*. For *M. lanceolata* and *M. balansae* there was no significant difference between the mixture with macerozyme and pectinase. For further experiments we decided to use the mixture with 1,5% cellulase R10, 0,5% macerozyme R10 and 0,5M mannitol for all species, except for *M. perlarius* for which we will use the mixture comprising 1,5% cellulase R10, 0,5% pectinase and 0,5M mannitol. The protoplasts from leaf material were smaller than callus protoplasts (Fig 7-4, measurements for *M. lanceolata* are shown in Table 7-1). For leaf protoplasts, viability was higher than 95% directly after isolation.

For *M. lanceolata* leaf material we also tested if isolation was possible within 5 hours (Table 7-1). There was no significant difference in protoplast yield when the leaves were incubated in the enzyme mixture during different time periods.

Hairy roots

The basic enzyme mixture (1.5% cellulase, 0.5% macerozyme and 0.5M mannitol) was tested directly with *Maesa lanceolata* hairy roots. Preliminary tests showed that the yield was very low and not reproducible. Moreover, protoplasts derived from the differentiated parts of the hairy roots did not survive enzyme treatment very well. In contrast, the root tips are composed of actively dividing cells that generated small protoplasts that persisted much better after the enzymatic treatment (results not shown). Hence, only root tips were used to obtain stable protoplasts. Because the hairy root cultures contained limited numbers of root tips and their isolation was very labor intensive, we increased the number of root tips in the hairy root culture by applying lateral root inducing growth regulators. Addition of IBA strongly stimulated lateral root formation in *Maesa* hairy roots. Treatment of hairy roots with IBA resulted in 7 root tips cm^{-1} while control hairy roots that were on medium without growth regulator showed on average 1.5 side roots cm^{-1} (Fig 7-8).

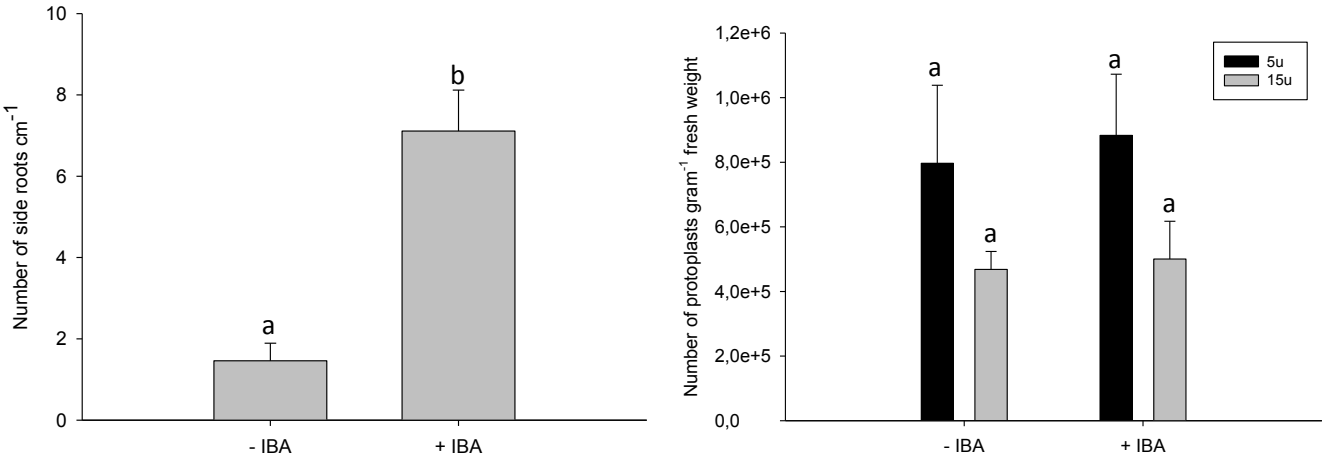

Fig 7-8 The effect of 5µM IBA was tested on the formation of side roots on *Maesa lanceolata* hairy roots (histogram **a**). Also the yield of protoplasts after a preculture of the hairy roots on medium with 5µM IBA was determined (histogram **b**).
Different letters indicate significant differences ($P < 0.05$); according to independent T-test. Significant differences were calculated between different hormone treatments (IBA or no IBA) (histogram **b**).

Treatment with IBA clearly enhanced lateral root formation along the entire roots system, which turned the complete root into good starting material for protoplast isolation. Protoplast isolation from roots treated with IBA yielded slightly more protoplasts than non-treated roots; however, this difference was not significant (Fig 7-8). Control hairy roots produced 7.8 $x10^5$ protoplasts $gram^{-1}$ fresh weight. IBA-treated roots produced 8.8 $x10^5$ protoplasts $gram^{-1}$ fresh weight after 5 hours of incubation in the enzyme solution. Incubation in the enzyme mixture for 15 hours yielded less protoplasts with both treatments compared to 5 hours incubation (Fig 7-8). The protoplasts isolated from hairy roots were much smaller than leaf or callus protoplasts and showed strong green fluorescence (Table 7-1, Fig 7-9).

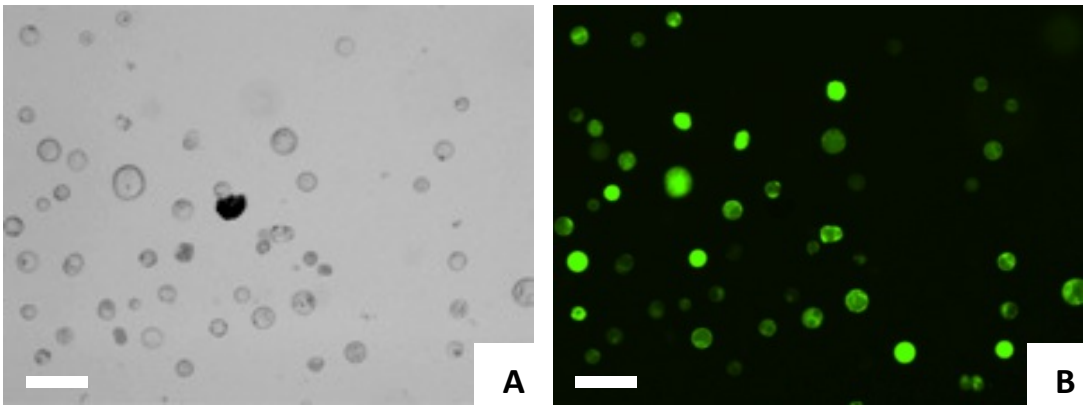

Fig 7-9 Protoplast isolated from *M. lanceolata* hairy roots. The hairy roots were pretreated with 5µM IBA to induce lateral root formation. The enzyme mixture consisted of 1.5% cellulase and 0.5% macerozyme. The protoplasts show strong green fluorescence. Scale bar = 50µm.

Although the difference in protoplast yield was not very large when treating roots with IBA or not, there was a significant difference in workload to obtain high protoplast numbers. Isolation of protoplasts treated with IBA was much more efficient and faster than isolation from non-treated hairy roots.

7.3.2 A method for automatic counting of protoplasts

Normally, a haemocytometer or counting chamber is used to determine the number of protoplasts in a suspension. Counting protoplasts manually is laborious and it becomes a limiting factor when many samples have to be processed. Therefore we tested if the Multisizer would be a faster and reliable alternative to count protoplasts. Protoplasts from leaves of four different *Maesa* spp. were used. The results are shown in Table 7-2.

Table 7-2 Comparison of the number of protoplasts counted manually with a haemocytometer or automatically with a Coulter Counter Multisizer^{TM}. Protoplasts isolated from leaves of four *Maesa* spp. were used.

Species	# protoplasts x 10^6 gram^{-1} fresh weight ± SD	
	Haemocytometer	Multisizer
M. argentea	6.3 ±0.2^a	2.5 ±1.3^b
M. balansae	8.3 ±0.7^a	6.1 ±0.4^b
M. lanceolata	5.2 ±1.4^a	5.2 ±0.9^a
M. perlarius	2.4 ±0.2^a	3.4 ±0.1^b

Different letters indicate significant differences ($P < 0.05$) between haemocytometer and Multisizer within one species; according to independent T-test.

Results show that there is a significant difference in the number of protoplasts counted with the haemocytometer and the Multisizer, except for the result obtained with *M. lanceolata*. Although the data showed statistical differences, the number of protoplasts counted was within the same order of magnitude, allowing the method to be used for comparative analysis. During the initial testing, we found that the concordance between haemocytometer and Multisizer results was higher when the quality (low amount of impurities due to lysed cells) of protoplasts was high. At optimal mannitol concentrations, the protoplast number was similar for both methods whereas at concentrations below or above the optimal, the protoplast numbers varied (Figure 7-10).

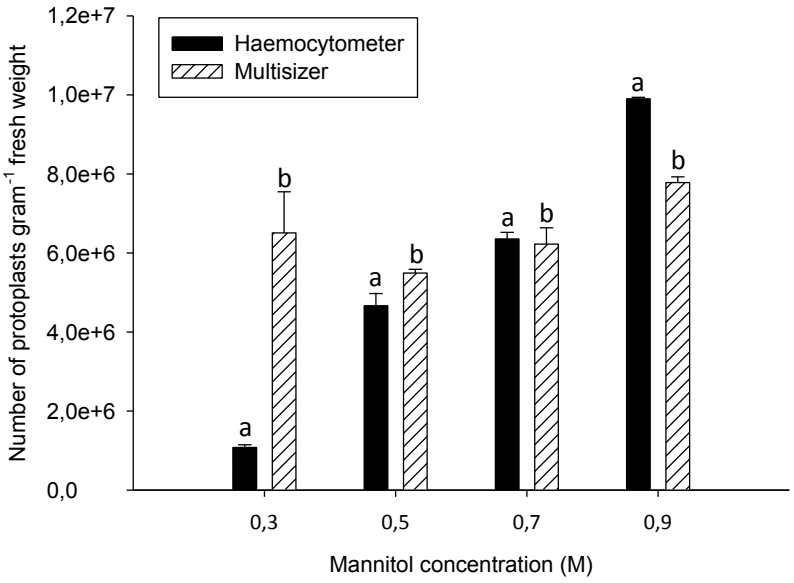

Fig 7-10 Protoplasts were isolated from *M. lanceolata* leaves using the basic enzyme mixture (1.5% cellulase and 0,5% macerozyme) with four different concentrations of mannitol (0.3M, 0.5M, 0.7M and 0.9M). The mannitol concentration is important for the morphology and viability of the protoplasts. Different letters indicate significant differences ($P < 0.05$) between haemocytometer and multisizer measurements within one mannitol concentration; according to independent T-test.

At a low mannitol concentration (0.3 M) the protoplasts swelled and eventually burst. As a result the samples were contaminated with cell debris that was not distinguished from intact cells through Multisizer measurements, generating overestimations of protoplast concentration. When high concentrations of mannitol (0,9M mannitol) were used, the protoplasts shrank. Under this condition, the Multisizer gave an underestimation probably because some of the protoplasts were smaller than the set limit. The optimal mannitol concentration of 0.5-0.7M was essential to obtain similar protoplast concentration measurements with the haemocytometer and Multisizer.

In addition to the tests to determine the accuracy of the Multisizer, we also performed an experiment to determine the reproducibility of the method (Fig 7-11).

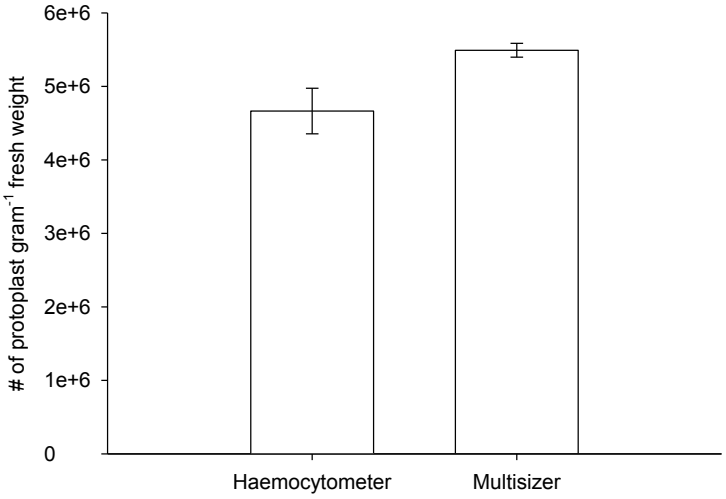

Fig 7-11 Average of triplicate counting of the number of protoplasts using the haemocytometer and the Multisizer.

The same sample was counted three times with the haemocytometer and three times with the Multisizer (Fig 7-11). The Multisizer data produced less variation than counting with the haemocytometer. This is reflected in the standard deviation, which is three times lower when using the Multisizer.

Consistently we observed an overestimation of the number of protoplast by means of Multisizer measurements. We think this was due to the presence of cell debris in the samples. Manual counting did not include these particles. When many samples need to be processed and an estimation of the concentration of protoplasts is sufficient, the Multisizer provides an interesting alternative that will lower the processing time.

7.3.3 Protoplast regeneration

For the regeneration of *Maesa* protoplasts many different protocols were tested and modulated in order to optimize them for the regeneration of *Maesa* protoplasts. The results of the main experiments are mentioned below, a complete list of all conditions tested can be found in Addendum II.

Protocol 1

An optimal medium for regeneration of protoplasts needs to be determined empirically, because different plant species and plant tissues have different nutritional requirements. However, there are some elements that need to be present in the culture medium in order to obtain cell wall regeneration and cell division; namely a salt formulation containing micro- and macroelements, vitamins, an osmoticum, a carbon source and growth regulators (Davey et al. 2005). The first medium tested for regeneration of *Maesa* protoplasts was based on MS salts with corresponding vitamins. MS and B5 formulations are very often used for protoplast media (Davey et al. 2005). As a carbon source we used 90mM sucrose, which was the same as for *Maesa* culture media. Beside a carbon source an osmoticum is added for osmotic protection until they form a new cell wall that can counteract the turgor pressure of the cytoplasm (Davey et al. 2005). Mostly a non-metabolisable sugar alcohol is used, such as mannitol or sorbitol. We used 0.5M mannitol and this concentration was reduced every three days or every week. Gradual reduction of the osmotic pressure is important for supporting cell division. Finally, also growth regulators are necessary for sustained cell division and growth and very often a combination of auxin with cytokinin is used (Davey et al. 2005). We tested a combination of 2.7µM NAA, 5µM 2,4-D and 4.4µM BA. In subsequent experiments, hormone concentrations and combinations were varied. In addition to auxins and cytokinins, also gibberellic acid was tested (Addendum II, Table 7-3).

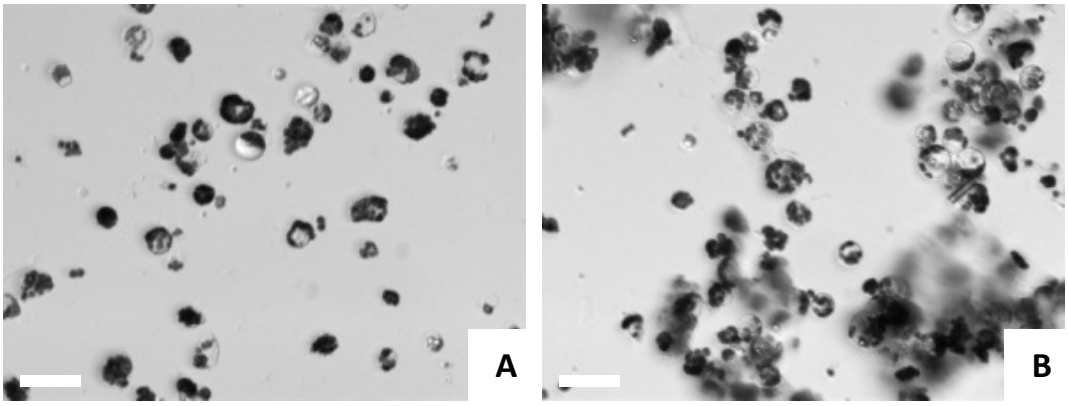

Fig 7-12 *Maesa balansae* protoplasts after four days in liquid regeneration medium comprising 2.5µM 2,4-D (**a**) and 5µM 2,4-D + 0.6µM GA3 (**b**), before the protoplasts were transferred to fresh culture medium with lower mannitol concentration. Scale bar = 50µm

These conditions were applied for *M. argentea* and *M. balansae* leaf protoplasts in liquid medium and on solid medium. For culture on solid medium, a nitrocellulose filter was placed on the agarose and the protoplasts were poured onto the filter, facilitating the transfer of protoplasts to fresh medium. After four days in liquid medium, the protoplast viability was very low and only few living protoplasts could be detected. Two examples are shown for *M. balansae* in Figure 7-12. Protoplasts on solid medium did also not develop further into microcallus.

Protocol 2

The presence of actively dividing cells in the protoplast culture can promote the mitotic division of protoplasts; this is called **nurse culture**. An efficient method for regeneration of recalcitrant banana protoplasts was developed by Assani and coworkers (2001, 2006) and was based on a **feeder layer** culture. Feeder layers are a type of culture where the nurse cells are immobilized in an agar, agarose or calcium-alginate layer. Protoplasts are then brought onto the feeder layer for regeneration. Nurse cells can be callus cells or protoplasts from the same or another species. For protoplasts derived from embryogenic *Musa acuminata* cell cultures, cell wall regeneration and sustained viability were observed for liquid culture and encapsulation in alginate beads. Though, mitotic activity was only found for protoplasts on a feeder layer of callus cells (Assani et al. 2001). This protocol was further optimized and it was found that a nitrocellulose filter between the feeder layer and the protoplasts increased frequency of microcallus formation significantly (Assani et al. 2006). For *Maesa* leaf and callus protoplasts a similar protocol, with few modifications, was tested. Callus cells of *M. balansae, M. lanceolata* and *M. perlarius* were mixed with an agarose medium consisting of 1x MS salts with vitamins, 0.4M glucose, 117mM sucrose, 0.5mM MES, 1.9mM KH_2PO_4, 0.5µM zeatin, 9µM 2.4-D and 1.2% (v/w) low melting agarose. The concentration of callus cells in the feeder layer was 10%. The same medium without callus cells was also tested for regeneration on solid medium. Protoplasts were suspended in culture medium with different hormone concentrations as in the feeder layer; namely 2.3µM zeatin, 1µM 2,4-D and 5.4µM NAA. Protoplasts were poured on the feeder layer/agarose layer, directly or on a nitrocellulose filter. The Petri dishes were kept in the dark at 25°C. These culture media were tested for *M. argentea, M. balansae* and *M. perlarius* leaf protoplasts and *M. balansae* and *M. perlarius* callus protoplasts. Both on feeder layers as on solid medium without nurse cells, the protoplasts did not show signs of cell division or colony formation. Furthermore, we did not observe differences between different species and explant material used. The cells used for the feeder layer did survive the treatment and started to form fresh callus after one week.

Assani et al. (2006) found that small changes in the agarose medium could have a strong effect on protoplast regeneration. Therefore we varied the hormone concentrations (both in the agarose medium as in the protoplast medium), more specifically, we changed 2,4-D concentration because 2,4-D proved to be the most important auxin for callus induction on *Maesa* leaves (Chapter 3). In addition, we changed the type of nitrocellulose filter and the preparation of the feeder layer (Addendum II, Table 7-4). These changes were tested for *M. argentea, M. balansae* and *M. lanceolata* leaf protoplasts, however, without any positive effect.

Immobilization of protoplasts in agar, agarose or alginate has several advantages; protoplasts remain separated, so single protoplasts are more easy to follow over time and it has been described that embedding protoplasts in solid medium supports cell wall regeneration and stimulates mitotic division (Davey et al. 2005). Leaf protoplasts of all four species were embedded in agarose drops (Fig 7-13). The culture medium was similar to the agarose medium used for feeder layers, however, we tested different concentrations of 2,4-D and zeatin and in a separate experiment we tried different types of salts and vitamins (Addendum II, Table 7-4). The agarose beads were submerged in liquid medium with the same composition as the agarose medium.

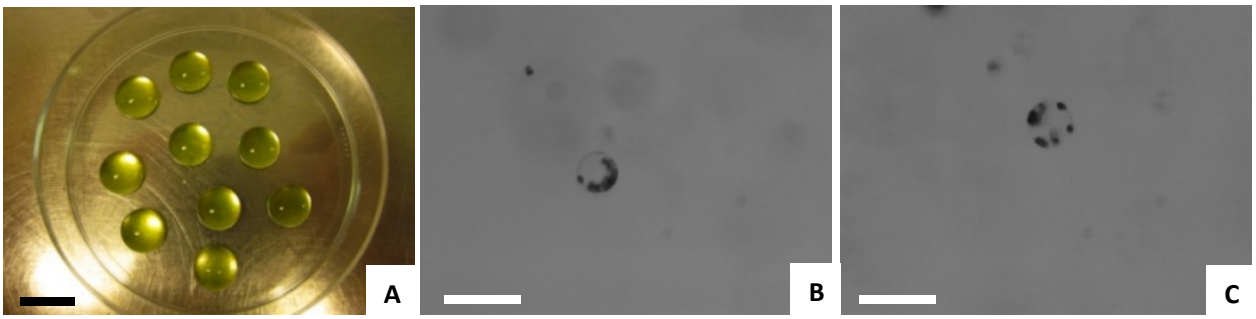

Fig 7-13 Agarose beads (before addition of liquid medium) with *M. argentea* protoplasts (**a**). *M. argentea* protoplasts after one week in agarose beads with 2.3µM zeatin + 5µM 2,4-D (**b**) and 2.3µM zeatin + 7.5µM 2,4-D (**c**). Scale bar = 1cm for picture **a** and 50µm for pictures **b** and **c**.

One week after immobilization in agarose beads, leaf protoplasts of all four species appeared normal (Fig 7-13), though, no cell divisions were noticed. We followed the protoplast cultures for over 3 weeks and no mitotic activity was observed.

In addition to feeder layers, solid medium and embedding techniques, we decided to test also regeneration in liquid medium, which is still mostly used for protoplast regeneration in general. A practical advantage of liquid culture was that the protoplasts were easier to monitor and to stain. In first

instance, the same medium as the protoplast medium was tested, however, with different concentrations of 2,4-D, NAA and zeatin. In total more than 80 different hormone combinations were tested for *M. argentea* leaf protoplasts (Addendum II, Table 7-4). For all hormones tested, we observed phenotypes that resembled protoplast fusion (Fig 7-14a). Spontaneous protoplast fusion was already described in 1972 by Withers and Cocking and the phenomenon was believed to be the result of the retention of plasmodesmatal connections because of the enzyme treatment (Withers and Cocking 1972). At the same time, however, we were able to detect cells that had formed a new cell wall (Fig 7-14b). Cell walls were detected using Calcofluor white staining, which can bind to the cell wall components callose and cellulose. There were no signs of cell division and no further development into microcallus was noticed.

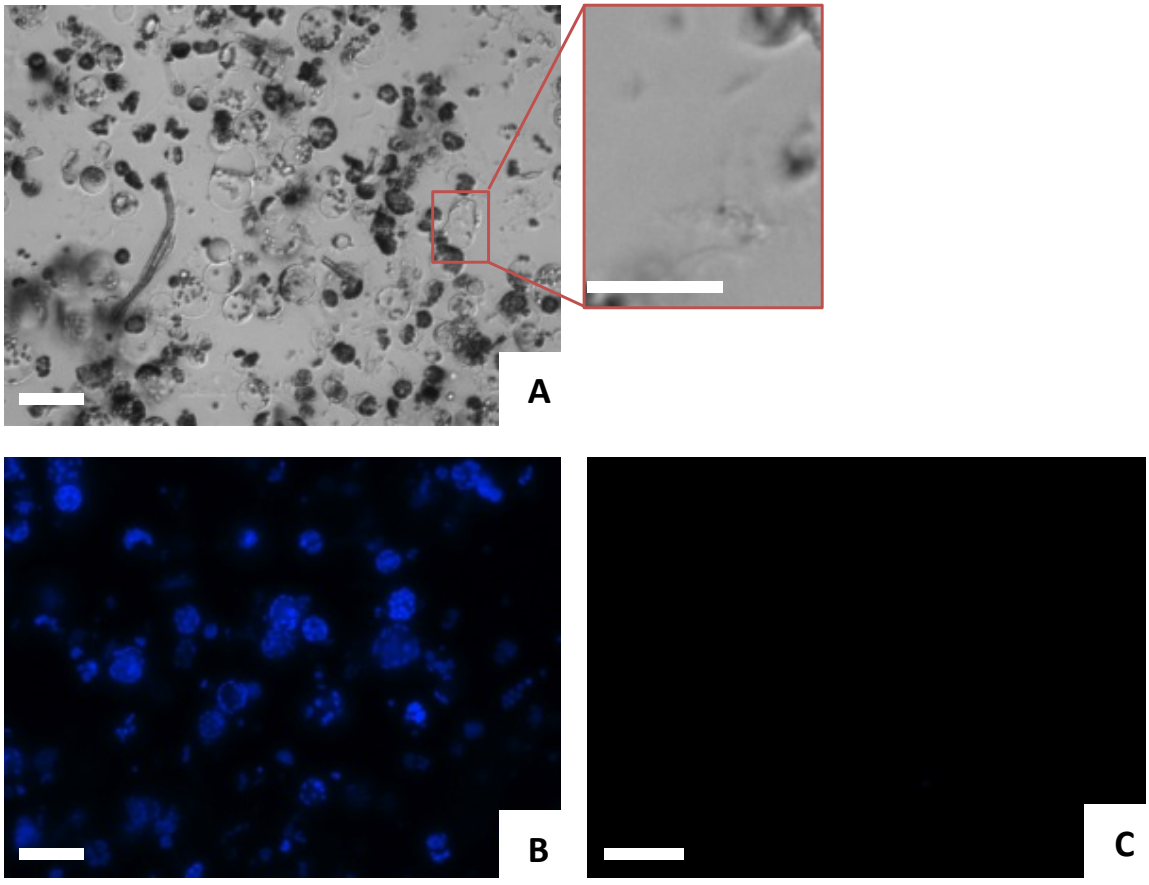

Fig 7-14 *M. argentea* protoplasts immediately after isolation (**b)** and after 1 week in liquid regeneration medium with 5.8µM zeatin + 5µM 2,4-D (**a**) and 3.5µM zeatin + 5µM 2,4-D (**c**). Protoplasts seem to fuse spontaneously during culture in the regeneration medium (**a**). Calcofluor white staining was performed to detect the formation of new cell walls. Immediately after isolation (**b)**, protoplasts do not have cell walls but some protoplasts did form a new cell wall in the first week of regeneration (**c**). Scale bar = 50µm for picture **a – b** and 25µm for the inset of picture **a** and picture **c**.

None of the concentrations and combinations of 2,4-D and zeatin stimulated cell division. Therefore we tested other phytohormones. The auxins 2.4-D, NAA, IBA, IAA were tried alone, in pair wise combination or in combination with the cytokinins kinetin, zeatin, TDZ and BA. In addition, we tested NPA, an auxin transport inhibitor. These conditions were tested for leaf protoplasts of all four species. Again we sometimes observed spontaneous fusion, however, no cell divisions were found and none of the protoplasts developed into microcallus.

In subsequent experiments, we kept the concentration of 2,4-D and zeatin constant, 7.5µM and 4.6µM respectively, and we varied the concentration of glucose and sucrose for *M. argentea* leaf protoplasts. In a separate experiment, we tested also other sugars and sugar alcohols as osmoprotectant and/or carbon source for *M. argentea* and *M. lanceolata*.

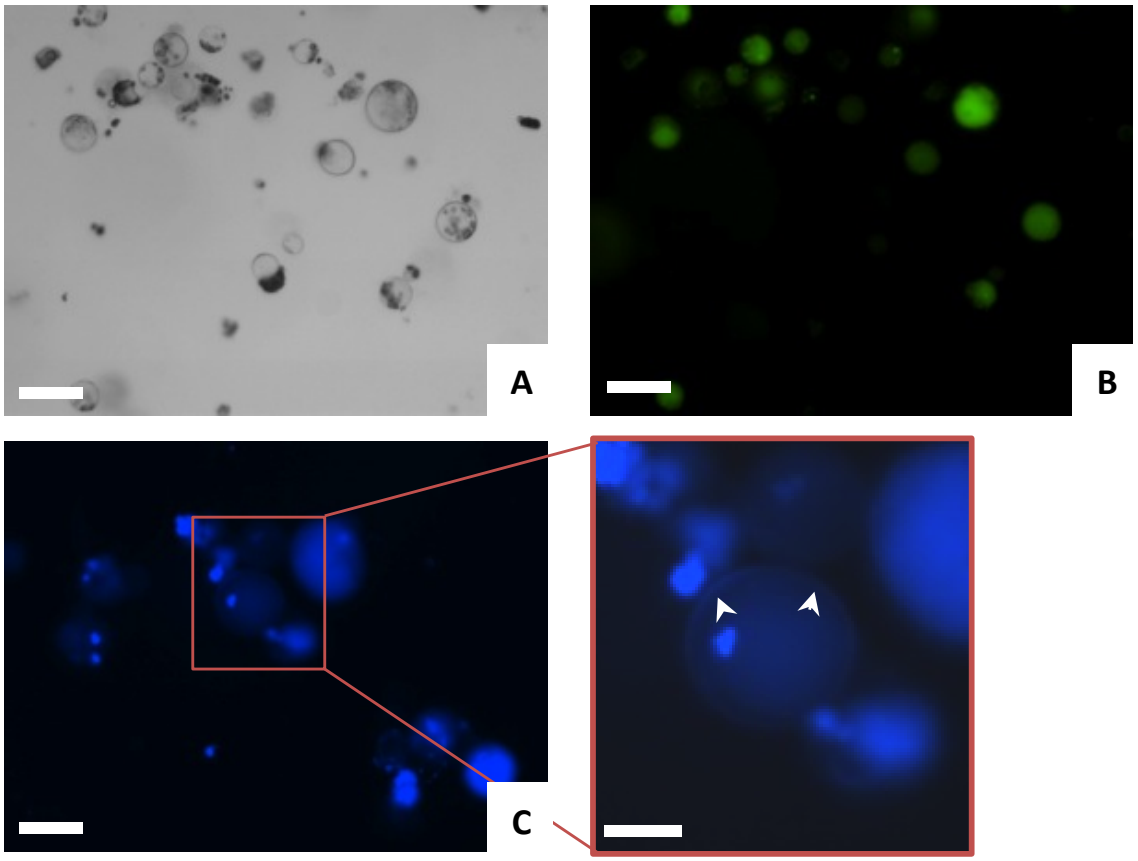

Fig 7-15 *M. argentea* protoplasts after 1 week in liquid regeneration medium met 0.6M glucose (**a + b**) and 0.2M sucrose + 0.4M glucose (**c**). FDA staining was performed to evaluate the protoplast viability (**a + b**) and CFW staining was used to visualize regenerated cell walls (**c**). Scale bar = 50µm for pictures **a, b** and **c**, and 25µm for the inset of picture **c**.

For both *M. argentea* and *M. lanceolata* we noticed a high viability using intermediate sugar concentrations after one week of culture, as determined with FDA staining (Fig 7-15 a-b). Fluorescein diacetate or FDA is a non-fluorescent derivative of fluorescein that can be transported across cell membranes. Living cells have esterases that can deacetylate the FDA molecule what results in the production of fluorescein. Accumulation of fluorescein can be detected using a fluorescence microscope. CFW staining revealed that some *M. argentea* protoplasts regenerated a cell wall (Fig 7-15c). For *M. lanceolata* protoplasts no cell wall was detected. When testing different types of sugars, it was found that a combination of 0.5M mannitol and 90mM sucrose was best for the survival of *M. argentea* and *M. lanceolata* leaf protoplasts. No cell division or microcallus formation was detected.

Furthermore, the influence of the salt formulations used for protoplast regeneration was tested in three experiments (Addendum II, Table 7-4). In the first experiment we compared medium based on MS, SH, B5 or KM salts. The concentrations of sugars (0.4M glucose and 117mM sucrose) and hormones (5µM 2,4-D and 2.3µM zeatin) were kept constant. This was tested for leaf protoplasts for all four *Maesa* species. For the second experiment we used *M. balansae* and *M. lanceolata* callus protoplasts to test the same conditions, but varying the hormone concentrations (1µM 2,4-D, 5.4µM NAA and 2.3µM zeatin). For a last experiment, we used only *M. argentea* leaf protoplasts and combined MS, B5, KM, SH, Heller and WPM based salt formulations with 90mM sucrose and 0.5M or 0.7M mannitol. The phytohormones 2,4-D and zeatin were used in a concentration of 5µM and 0.5µM, respectively. In view of viability of protoplasts, we found that MS and B5 salts were best. For none of the tested conditions cell division was observed. Cell wall formation was not examined in these experiments.

Finally, we did some small tests to examine the effect of light, temperature, Pluronic (a surfactant) and the coating of plates with foetal calf serum (Addendum II, Table 7-4). *M. argentea* and *M. lanceolata* leaf protoplasts were placed in different growth rooms with different light and temperature conditions; 25°C dark, 25°C 16/8 light/dark and 28°C 16/8 light/dark. From the pictures in Figure 7-16 it is clear that light and a combination of light and higher temperatures had a negative effect on the protoplast. After one week, almost no viable protoplasts were found except for the protoplasts incubated in the dark at 25°C. However, these protoplasts did not regenerate.

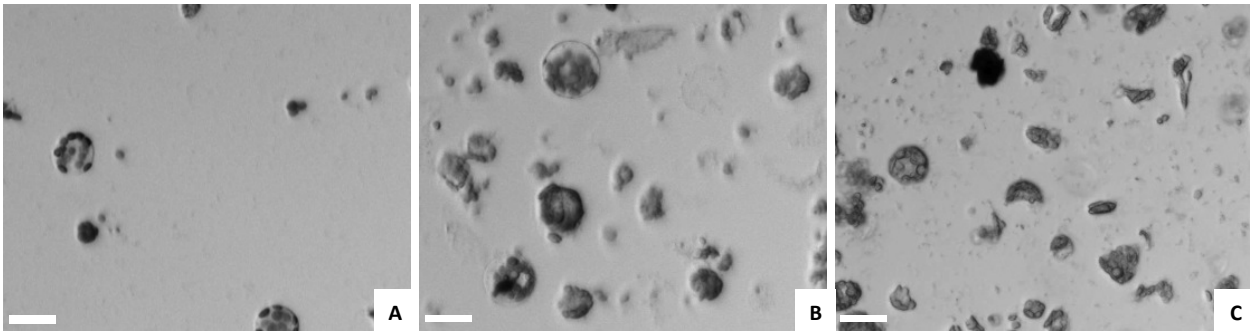

Fig 7-16 *M. lanceolata* protoplasts after one week in liquid medium, incubated in the dark at 25°C (**a**), in the light at 25°C (**b**) and in the light at 28°C (**c**). Scale bar = 25µm.

Pluronic F-68 is a polyoxyethylene-polyoxypropylene copolymer and has the properties of a non-ionic surfactant. It is often used as a non-toxic, low cost cell protecting agent during culture of both animal and plant cells (Lowe et al. 2001). Moreover, Pluronic has been used in protoplast cultures to enhance mitotic division (Davey et al. 2005). For *M. lanceolata* leaf protoplasts, addition of 0.1% Pluronic did not induce protoplast division. Pluronic was also did not protect the protoplasts that were incubated in light conditions.

Coating the plates (in our case with fetal calf serum, FCS) before transferring protoplasts prevents the protoplasts from sticking to the bottom. FCS is also included in basic medium formulations for animal cell culture and therefore, we believed that it could also have a positive effect on protoplast suspensions. We have tested coating with 100% and 5% FCS and we found that 5% FCS had a positive effect on viability of the protoplasts compared to the protoplasts in non-coated plates and plates coated with 100% FCS. Coating with FCS did not affect cell division or development.

Protocol 3

In our lab, successful protoplast regeneration was achieved for three different varieties of *Cichorium intybus*, based on a protocol described in literature (Nenz et al. 2000). The original protocol of Nenz and co-workers was based on a liquid culture. After two days in liquid medium, some protoplasts were maintained in liquid medium, some were transferred onto solid medium and others were embedded in calcium-alginate drops. Stratification on solid medium and embedding in alginate appeared to be better culture techniques than liquid medium for regeneration of *Cichorium* protoplasts (Nenz et al. 2000). However, liquid culture did prove to be very efficient for the regeneration of *C. intybus* varieties tested in

our lab (Barua et al. *unpublished results*). Because of the successful application of this protocol in our lab, we implemented it also for *Maesa* species.

M. lanceolata protoplasts were suspended in liquid medium consisting of 1x Heller salts with vitamins, 10mM sucrose, 0.4M mannitol, 4.5µM BA and 10µM NAA. Some of the protoplasts were kept in liquid medium and others were transferred onto solid medium with the same composition. In two experiments, Pluronic was also added to the culture medium in a concentration of 0.1% or 0.5% (Addendum II). In addition we also tried embedding the protoplasts in agarose beads, agarose layers or calcium alginate beads with the same medium composition. Unfortunately, for none of the experiments we observed cell division or microcallus formation.

Protocol 4

The last protocol that we have tested was based on a protocol for regeneration of protoplasts from callus of *Panax ginseng* (Han et al. 2009). *Maesa lanceolata* protoplasts were brought in liquid medium containing 1x MS salts with vitamins, 0.5M glucose, 30mM sucrose and 5µM 2,4-D. In addition, the medium was supplemented with 27µM glycine, 1mM glutamine and 500mg/l casein hydrolysate (Addendum II). However, this culture medium was not suited for regeneration of *M. lanceolata* protoplasts as there were no living protoplasts observed after only three days of culture.

7.3.4 Preliminary tests for the transient transformation of *Maesa lanceolata* protoplasts

Because they lack a cell wall, protoplasts are more amenable for protoplast transformation than normal plant cells. The plasma membrane has fluid mosaic characteristics and DNA uptake can be induced by chemical and/or physical procedures. Treatment of protoplast-plasmid mixtures with polyethylene glycol (PEG) and/or electroporation is the most exploited approach to induce DNA uptake into protoplasts (Davey et al. 2005). For transformation of *Maesa lanceolata* protoplasts we adapted a protocol for PEG mediated transient gene expression in *Arabidopsis* mesophyll protoplasts (Yoo et al. 2007).

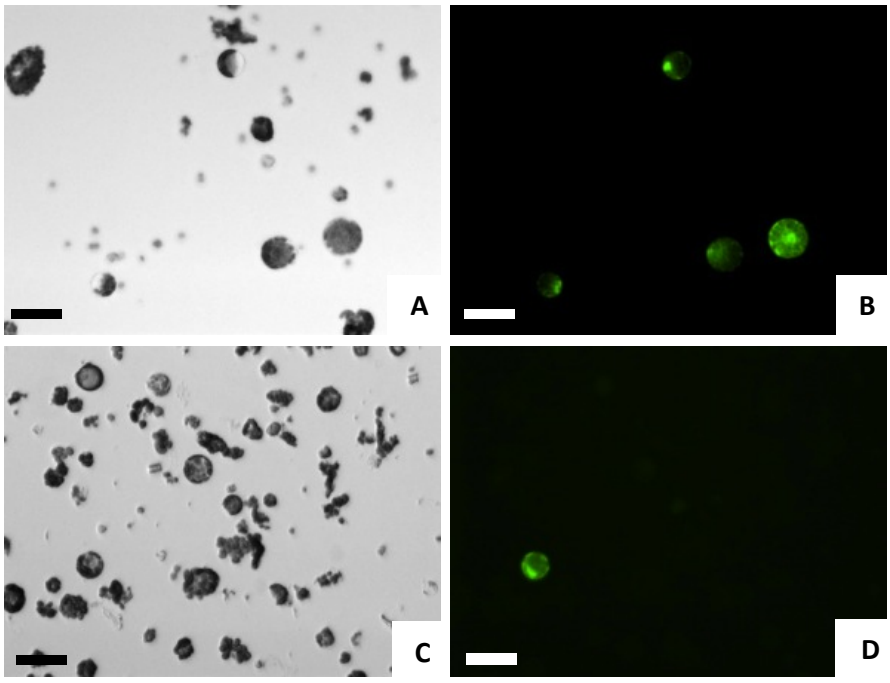

Fig 7-17 *Arabidopsis thaliana* (**a** – **b**) and *Maesa lanceolata* (**c** – **d**) leaf protoplasts showing transient expression of an eGFP gene 10 hours after PEG mediated transformation.

Arabidopsis thaliana and *Maesa lanceolata* leaf protoplasts were transformed with a pK7WG2 vector comprising a p35S promoter followed by a cytoplasmic eGFP gene and a T35S terminator. Protoplasts were incubated in a DNA-PEG solution for 5 minutes. We used 15µg DNA for 100µl cells with a concentration of approximately 2 x 10^5 protoplasts per ml and 20% PEG 4000. Transformation efficiency was evaluated after 10 hours. For *A. thaliana* 36% of the protoplasts were transformed, however, for *M. lanceolata* only 5% of the protoplasts showed eGFP expression. Therefore, more tests will be necessary to optimize the protocol for transformation of *Maesa* protoplasts.

7.4 Discussion

Genetic recombination is often used for the production of important microbial products such as antibiotics. Moreover, strain improvement programs routinely include protoplast fusion between different (mutant) lines. Hybridization of different microbial strains can result in higher yields of microbial products, synthesis of new products and improved characteristics of the microorganism (e.g. improved growth, higher sporulation and better seed growth) (Adrio and Demain 2006). In plants, the technique of protoplast fusion has mainly been used as a breeding technique to overcome natural

crossing barriers (Davey et al. 2005). However, some articles report quantitative and qualitative changes in secondary metabolite production in somatic hybrids. Laurila et al. (1996) showed that protoplast fusion between *Solanum brevidens* and *Solanum tuberosum* can result in the production of a novel secondary metabolite that is not present in the parental species (Laurila et al. 1996). Similar results were published recently by Saverese and co-workers when analysing the glycoalkaloid content of somatic hybrids of *Solanum tuberosum* and *Solanum bulbocastanum* (Savarese et al. 2009). In addition, asymmetric hybrids through protoplast fusion between *Panax ginseng* and *Daucus carota* contained variable concentrations of ginsenoside Rb1 and showed other unidentified secondary metabolites in the HPLC chromatogram (Han et al. 2009).

In this study we first developed and optimized a method for isolation of protoplasts from leaves, callus and hairy roots of all four *Maesa* species. For callus material, the enzyme mixture comprising 1.5% cellulase, 0.5% macerozyme and 0.5M mannitol proved to be most successful for all four species. There were, however, large differences in yield of the different species. Isolation was most efficient for *M. balansae* and *M. perlarius* with 1.7 x 10^6 and 1.4 x 10^6 protoplasts gram^{-1} fresh weight, respectively. For *M. lanceolata* enzyme treatment of callus material yielded 7.7 x 10^5 protoplasts gram^{-1} fresh weight. Finally, for *M. argentea* only 6.6 x 10^4 protoplasts gram^{-1} fresh weight were isolated. These results are in line with what is already published for other plants. Callus of *Echinacea augustifolia* yielded 5.0 x 10^5 protoplasts gram^{-1} fresh weight and callus of *Agapanthus praecox* yielded 0.8 – 1.5 x 10^6 protoplasts gram^{-1} fresh (Nakano et al. 2003; Zhu et al. 2005). Callus material of the woody plant species *Robinia pseudoacacia* produced at maximum 3.5 x 10^5 protoplasts gram^{-1} fresh weight after enzymatic treatment (Kanwar et al. 2009).

In general, leaf material yielded more protoplasts compared to callus, using the same enzyme mixtures. For *M. argentea*, *M. balansae* and *M. perlarius* the enzyme mixture consisting of 1.5% cellulase, 0.5% pectinase and 0.5M mannitol was most efficient, *M. perlarius* leaves yielded the highest number of protoplasts using 1.5% cellulase, 0.5% pectinase and 0.5M mannitol. Using *in vitro* leaves as explant material, less variation in protoplast yield between different species was noticed. *M. lanceolata* and *M. argentea* produced 6.9 x 10^6 and 5.2 x 10^6 protoplasts gram^{-1} fresh weight, respectively. *M. perlarius* and *M. balansae* yielded 2.8 x 10^6 and 2.1 x 10^6 protoplasts gram^{-1} fresh weight, respectively. The highest yield of protoplasts was 6.1 x 10^6 g^{-1} fresh weight when starting from leaf material. These protoplast yields are better or comparable to the results described in literature. For example, for *Calibrachoa* leaves 1.1 – 4.6 x 10^6 protoplasts g^{-1} fresh weight and for *Petunia* 1.0 – 3.6 x 10^6 protoplasts g^{-1} fresh weight was

achieved (Meyer et al. 2009). Leaves of *Spathiphyllum wallisii* and *Anthurium scherzerianum* yielded respectively 9 x 10^5 and 7 x 10^4 protoplasts gram^{-1} fresh weight (Duquenne et al. 2007). For *Robinia pseudoacacia* it was also noticed that more protoplasts could be isolated from leaf than from callus material, namely 9.5 x 10^5 compared to 3.5 x 10^5 protoplasts gram^{-1} fresh weight, respectively (Kanwar et al. 2009).

Very few studies report on the isolation of protoplasts from hairy roots (Sevon et al. 1997; Sevon et al. 1998). Yet, hairy roots are good source material for the production of transgenic protoplasts expressing marker genes like GFP which could be helpful for protoplast fusion experiments and tracking the regeneration process. The isolation of protoplasts from hairy root cultures did not produce sufficiently high yield for protoplast for fusion or transformation experiments. This was probably due to the relatively low representation of meristematic cells with digestible cell walls. To obtain a good protoplasts yield, root tips must be separated and collected for enzyme treatment. The isolation of the root tips is very laborious and requires the growth of large quantities of hairy root cultures. By means of application of the lateral root-inducing hormone IBA, the number of root tips increased and allowed high yield protoplast isolation from hairy root cultures directly without isolating root tips. Although plants contain less IBA than IAA *in vivo*, IBA is more efficient for formation of lateral roots, hence its use in agricultural applications (De Klerk et al. 1999; Hartmann et al. 1990). Treatment of hairy roots with IBA resulted in 5 times more lateral roots cm^{-1} compared to non-treated controls. Isolation of protoplasts from treated and non-treated hairy roots yielded respectively 8.8 x 10^5 and 8.0 x 10^5 protoplasts gram^{-1} fresh weight. Although the yield of protoplasts was similar, the process of protoplast isolation was greatly facilitated when hairy roots were treated with IBA.

In 1975 it was already mentioned that protoplasts of 'some' species will regenerate a new cell wall and will divide to produce a callus from which plants can be regenerated (Thomas and Davey 1975). Later on it became clear that some tissues, mainly from cereals, perennial woody plant species and legumes, were recalcitrant regarding protoplast regeneration (Cutler et al. 1991; Papadakis and Roubelakis-Angelakis 2002; Siminis et al. 1993). It seems that also *Maesa* protoplasts are recalcitrant, as we did not observe cell division with none of the tested conditions. It is possible that the nutritional requirements were not suitable to obtain mitotic division; however, intensive efforts were made to optimize the culture medium for *Maesa* protoplasts. We varied the salt formulation, carbon source, osmoprotectant, hormone type/concentration/combination, culture type and additional factors like addition of a surfactant, incubation conditions and coating of the plates with foetal calf serum. Although we believe

that the inadequate medium composition for protoplast culture may contribute to the recalcitrance, it is unlikely to be the major factor. Another important factor that is not yet completely explored in this work is the protoplast density. Small changes in protoplasts concentrations in the regeneration medium could have a drastic effect on protoplast regenerating capacity (Davey et al. 2005). It is striking, though, that intact leaf cells of all four species were still able to make callus and shoots and callus material of three species could regenerate to shoots (Chapter 3). Thus, leaf and callus cells were not terminally differentiated and were still able to re-enter the cell cycle. However, it is also reported that only protoplasts of embryogenic calli could regenerate. Overall, it seems that treatment with enzymes and removal of the cell wall changed the cells in such a way that they lost their mitogenic potential. An interesting hypothesis on the recalcitrance of cereal protoplasts is proposed by Cutler and co-workers (1991). During cell wall digestion deleterious changes are initiated in the plasma membrane. For example, there are changes in plasma membrane protein phosphorylation and the membrane potential is depolarized which changes the transport properties of the membranes (Blowers et al. 1988; Morris et al. 1981). These are common phenomena during protoplast isolation and non-recalcitrant cells will react to these changes through induction of a wound response (Bolwell 1988). However, recalcitrant cells probably do not have a normal wound response and therefore, these changes will lead to lipid peroxidation and oxidative stress (Cutler et al. 1989; Papadakis and Roubelakis-Angelakis 2002). Moreover, a combination of membrane perturbations and oxidative stress can cause changes in cytoskeleton. Several studies already indicated that microtubules were deficient in recalcitrant protoplasts (Hahne and Hoffmann 1985; Meijer and Simmonds 1988; Wang et al. 1989). Abnormalities in the cytoskeleton and cell wall formation will eventually prevent mitosis (Hahne and Hoffmann 1985; Maiato 2010; Schilderentschler 1977). For *Maesa* protoplasts, cell wall formation was observed only for very few *M. argentea* protoplasts. The spontaneous fusion phenomenon observed for all species under many different conditions is evidence that cell walls were not formed (Withers and Cocking 1972). The inability of *Maesa* protoplasts to regenerate cell walls is an indication that they do not respond in a correct way to enzymatic treatment, which leads to mitotic arrest.

Transformation of protoplasts has been successful in a number of plant species and DNA uptake into protoplasts has been especially important in transforming plants that are not amenable to other methods of gene delivery, specifically *Agrobacterium* mediated methods (Davey et al. 2005; Rakoczy-Trojanowska 2002). Protoplast transformation offers a system for investigating transient gene expression, which can be very useful in functional genomics analysis. Protoplast transient expression assays have been widely used for the identification of key regulators and the elucidation of molecular

mechanisms underlying intracellular transduction pathways (Yoo et al. 2007), for example jasmonate signalling (De Sutter et al. 2005), MAP kinase signalling in innate immunity (Asai et al. 2002), cytokinin signal transduction (Hwang and Sheen 2001) and gibberellin/abscisic acid signalling pathway (Zentella et al. 2002). Stable protoplast transformation has also been obtained for several species, including maize (Lyznik et al. 1989), barley (Tiwari et al. 2001), rice (Rao et al. 1995) and citrus (Omar and Grosser 2008). For *M. lanceolata* a protocol for transformation of *Arabidopsis thaliana* mesophyll protoplasts was tested (Yoo et al. 2007). Transformation frequencies were 36% and 5% for *A. thaliana* and *M. lanceolata* respectively. It is clear that the protocol needs further optimization for effective transformation of *M. lanceolata* protoplasts. A transient expression system in *M. lanceolata* protoplasts could be used to quickly test the functionality of new constructs but also to investigate the activity of the putative saponin biosynthesis genes that were described in Chapter 5.

In conclusion, a highly efficient protocol for the isolation of protoplasts from different types of *in vitro* cultures of *M. argentea, M. balansae, M. lanceolata* and *M. perlarius* was established. In addition, we investigated an automatic method for counting protoplasts. Counting with the Coulter Counter Multisizer^{TM} could drastically decrease processing time when many samples have to be processed. Regarding protoplast regeneration, we were not able to induce mitotic division, despite the numerous conditions tested. This led us to conclude that *Maesa* protoplasts are recalcitrant. One possible reason for the apparent mitotic arrest of the protoplasts is the lack of cell wall regeneration. Further investigation will be needed to find conditions that allow the formation of cell walls. Transient transformation of *M. lanceolata* protoplasts was successful, however, only very low transformation frequencies were found so optimization of the used protocol is still necessary.

7.5 Materials and methods

7.5.1 Plant material

M. argentea, M. balansae, M. lanceolata and *M. perlarius* were cultured *in vitro* as described in the Materials and methods section in Chapter 2.
Induction and culture of *M. lanceolata* hairy roots is described in detail in the Materials and methods section of Chapter 5.

7.5.2 Protoplast isolation

General procedure for protoplast isolation

Starting material for protoplast isolation was 500 mg of *Maesa* callus, 100 mg of *Maesa in vitro shoots* or 100 mg of *Maesa lanceolata* hairy roots. The plant material was incubated in an enzyme solution for 5 or 15 hours in the dark at 25°C and on a rotary shaker at 50 rpm. After incubation, the protoplast – enzyme mixture was filtered through a 40µM nylon filter (BD Biosciences Europe) and centrifuged for 10 minutes at 500 rpm. The supernatant was discarded and the pellet was resuspended in a washing solution (1x MS salts and the same concentration of mannitol/sorbitol that was used during the isolation) and centrifuged for 5 minutes. After another wash following the above procedure, the centrifugation was repeated. Finally the protoplasts were resuspended in 1ml of washing solution.

Protoplast isolation from callus material

In order to find the best conditions for protoplast isolation, different enzyme mixtures were tested on *Maesa lanceolata* callus. The mixture consisted of enzymes, a sugar alcohol (mannitol or sorbitol) and 5ml of distilled water. Four different enzymes were used in the given concentrations; 1%, 1.5% or 2% (w/v) cellulase onnozuka RS (Duchefa Biochemie BV, The Netherlands), 1.5% (w/v) cellulase Onnozuka R-10 (Duchefa Biochemie BV, The Netherlands), 0.5%, 1% and 1.5% (w/v) macerozyme R-10 (Duchefa Biochemie BV, The Netherlands) and 0.05% or 0% (w/v) pectolyase Y-23 (Duchefa Biochemie BV, The Netherlands). Mannitol was used in a concentration of 0.5 M, 0.6 M, 0.7 M or 0.8 M; sorbitol was used in a concentration of 0.5 M, 0.6 M or 0.7 M. Subsequently, three different enzyme mixtures were tested for callus material of all four *Maesa* spp. (1) 1.5% (w/v) cellulase Onnozuka R-10 + 0.5% (w/v) macerozyme R-10, (2) 1.5% (w/v) cellulase Onnozuka R-10 + 0.5% (w/v) pectolyase Y-23 and (3) 1.5% (w/v) cellulase Onnozuka R-10 + 0.5% (w/v) pectinase (Sigma). All enzyme mixtures were combined with 0.5 M mannitol. Enzyme mixture 1 was tested also on *Maesa lanceolata* callus induced on hairy roots.

Protoplast isolation from leaf material

Maesa leaf material was sliced into very small strips (1 – 2 mm) before incubation in the enzyme mixture. For leaf material three enzyme mixtures were tested; (1) 1.5% (w/v) cellulase Onnozuka R-10 + 0.5% (w/v) macerozyme R-10, (2) 1.5% (w/v) cellulase Onnozuka R-10 + 0.5% (w/v) pectolyase Y-23 and

(3) 1.5% (w/v) cellulase Onnozuka R-10 + 0.5% (w/v) pectinase. All enzyme mixtures were combined with 0.5 M mannitol.

Protoplast isolation from hairy root material

To enhance the yield of protoplasts when using *Maesa lanceolata* hairy roots as starting material, the roots were treated with 5 µM indole-3-butyric acid (IBA) one week before protoplast isolation. The root tips of the hairy roots were removed and placed on medium containing IBA or on medium without growth regulator. After 24 hours the hairy roots were transferred to basal SH medium without any growth regulators. One week later, 100mg of the hairy roots was chopped into small pieces and transferred to 5 ml of enzymatic solution. The enzyme mixture for protoplast isolation from hairy roots consisted of 1.5% (w/v) cellulase Onnozuka R-10, 0.5% (w/v) macerozyme R-10 and 0.5M mannitol.

Viability staining

For trypan blue (TB) staining, protoplasts were mixed with trypan blue solution (Sigma) in a 1/1 ratio and incubated during 1 minute.

Fluorescein diacetate (FDA) was dissolved in DMSO to make a stock solution of 5mg/ml. From this stock solution 0.25µl was added to 500µl of protoplast suspension. The solution was immediately centrifuged for 5 minutes at 500 rpm in a swinging bucket centrifuge. The supernatans was removed and the pellet was resuspended in washing buffer or regeneration medium.

7.5.3 Protoplast counting

A Fuchs-Rosenthal haemocytometer was used to count protoplasts manually. The number of protoplasts was counted in four squares and the amount of protoplasts in one ml was calculated using the following formula: (# counted protoplasts * 1000) / (counted area x depth of the chamber) = # protoplasts/ml.

Automatically counting of protoplasts was performed using a Coulter Counter Multisizer^{TM} 3 (Beckman Coulter, Inc.). For protoplast measurements, 50 µl of protoplasts were suspended in 10ml of washing solution comprising mannitol (in a concentration that was dependent on the experiment) and 1x MS salts. The Multisizer counted all particles in 2 ml of this solution. The number of protoplasts in one ml could be easily calculated by multiplying the number of protoplasts by 100.

7.5.4 Protoplast regeneration

Protocol 1

Protoplasts were suspended in culture medium consisting of 1x MS salts including vitamins, 90mM sucrose, 0.5M mannitol, 5µM 2.4-D, 4.4µM BA and 2.7µM NAA. For regeneration on solid medium, the protoplasts were poured on solid medium with the same composition (with addition of 0.7% (w/v) agar)

on a nitrocellulose filter (Millipore, type AA, 0.8µm). The mannitol in the culture medium was reduced gradually; for protoplasts on solid medium, the filter was placed on fresh medium with a lower mannitol concentration and for protoplasts on liquid medium, the medium was replaced with fresh medium with a lower mannitol concentration. The mannitol concentration in the medium was lowered to zero in four steps (0.5M → 0.3M → 0.1M → 0). Protoplasts were incubated in the dark at 25°C. Variations on this basic protocol are shown in Table 7-3.

Table 7-3 Medium components that were varied for protoplast regeneration using protocol 1

Fresh medium with lower mannitol concentration		Every three days – every week
Hormones	2.4-D	0 – 2.5µM – 5µM – 10µM
	GA3	0 – 0.3µM – 0.6µM – 0.9µM

Protocol 2 (Assani et al. 2001, 2006)

Friable calli, 1 week post subculturing, were cut into small pieces and approximately 2.5 ml of callus material was transferred to 25 ml of double concentrated A2 medium. Medium A2 consisted of 1x MS salts including vitamins, 0.4M glucose, 117mM sucrose, 0.5mM MES, 1.9mM KH_2PO_4, 0.5µM zeatin and 9 µM 2,4-D. The cell suspension was mixed with 25 ml of double concentrated agarose mixture (with a final agarose concentration of 1.2%) when the temperature of the agarose was decreased to +/- 30°C. The concentration of feeder cells in the agarose layer was 10%. Aliquots of the final mixture were poured into small Petridishes. After solidification, the medium was covered with a nitrocellulose filter (Millipore type AA 0.8µm). The feeder layers were prepared one day beforehand.
Protoplasts were suspended in B medium, comprising 1x MS salts including vitamins, 0.4M glucose, 117mM sucrose, 0.5mM MES, 1.9mM KH_2PO_4, 2.3µM zeatin, 1µM 2,4-D and 5.4µM NAA. 0.5ml of the protoplast suspension was gently transferred to the feeder layer. The protoplasts were incubated in the dark at 25°C. Variations on this protocol are shown in Table 7-4.

Table 7-4 Medium components that were varied for protoplast regeneration using protocol 2 in different culture types

Feeder layer/solid medium		
Filter type		Type AA (0.8µm) – Type GW (0.22µm) – none
Incubation of feeder layer		1 day – 1 week
Hormones (Medium A2):	2,4-D	0 – 0.5µM – 5µM – 9µM
Hormones (Medium B):	2,4-D	0 – 1µM – 5µM
	NAA	0 – 0.54µM – 5.4µM
Agarose beads		
Hormones (Medium A2)	2,4-D	0 – 2.5µM – 5µM – 7.5µM
	Zea	0 – 2.5µM – 4.6µM
	NAA	0 – 0.5µM
Salts		MS – SH – B5 – KM
Liquid medium		
Hormones (Medium B)	2.4-D	0 – 1.25µM – 2.5µM – 3.75 µM – 5µM – 6.25µM – 7.5µM – 8.75µM – 10µM – 11.25µM – 12.5µM
	NAA	0 – 0.5µM – 2.7µM – 5µM
	Zea	0 – 0.5µM – 1.2µM – 2.3µM – 3.5µM – 4.6µM – 5.8µM – 6.9µM
	IBA	0 – 5µM
	IAA	0 – 3µM – 5µM – 6µM – 9µM – 12µM
	Kin	0 – 0.5µM
	BA	0 – 4.5µM
	NPA	0 – 5µM
	TDZ	0 – 0.5µM
	GA3	0 – 1.5µM – 3µM – 4.5µM – 6µM
	GA4-7	0 – 1.5µM – 3µM – 4.5µM – 6µM
Sugars	Glucose	0 – 0.2M – 0.3M – 0.4M – 0.5M – 0.6M – 0.7M – 0.8M – 1M
	Sucrose	0 – 0.08M – 0.2M – 0.4M – 0.6M – 0.8M – 1M
	Mannitol	0 – 0.4M – 0.5M – 0.7M
	Sorbitol	0 – 0.5M – 0.7M
	Xylose	0 – 0.4M – 0.5M – 0.6M – 0.9M
	Fructose	0 – 0.3M – 0.5M – 0.7M
Salts		MS – SH – B5 – KM – Heller – WPM
Pluronic		0 – 0.1 – 0.5%
Coating		0 – 5% FCS – 100% FCS
Temperature		25°C – 28°C
Light conditions		Dark – 16/8 day/night

Protocol 3 (Nenz et al. 2000)

The basic culture medium used for the third protocol consisted of 1x Heller salts including vitamins, 10mM sucrose, 0.4M mannitol, 4.5µM BA and 10µM NAA. Pluronic was added in some experiments in a concentration of 0.1 or 0.5%. This medium composition was tested for protoplast regeneration in liquid medium and on solid medium (+ 7% (w/v) agar). Protoplasts were also embedded in agarose layers,

agarose beads (both + 1.2% (w/v) agarose) and calcium-alginate beads. For encapsulation in calcium-alginate beads, the protoplasts were suspended in 1ml of regeneration medium. This suspension was gently mixed with an equal volume of alginate solution. The alginate solution comprised 30g/l sodium-alginate (Fluka BioChemika), 0.4M mannitol and 7.75 g/l MES. Afterwards, the protoplast-alginate suspension was dropped in a calcium solution, consisting of 50mM $CaCl_2.2H_2O$ and 0.4M mannitol. After 30 minutes, the calcium solution was removed and the beads were washed 2-3 times with regeneration. Eventually, the beads were suspended in 6ml of regeneration medium. Protoplasts were incubated at 25°C in the dark.

Protocol 4 (Han et al. 2009)

Callus protoplasts were suspended in liquid regeneration medium comprising 1x MS salts including vitamins, 0.5M glucose, 30mM sucrose, 5µM 2,4-D, 27µM glycine (Sigma), 1mM glutamine (Sigma) and 500mg/l casein hydrolysate (Sigma).

7.5.5 Transient transformation of *Maesa* and *Arabidopsis*

First, the enzyme solution was prepared (10ml): 3.9ml H_2O was mixed with 5ml 0.8M mannitol, 0.1ml 2M KCl and 1ml 0.2M MES. This suspension was heated for 2 minutes at 70°C. The enzymes were weighed and added; 1.5% (w/v) cellulase R-10 and 0.4% (w/v) macerozyme. After dissolving the enzymes, the mixture was heated for 10 minutes at 55°C. The enzyme solution was cooled on ice to room temperature and 0.1ml 1M $CaCl_2$, 3µl β-mercaptoethanol and 0.1ml 10% BSA were added.
Leaves were cut in to 0.5 – 1mm strips with a sharp razorblade and were submerged in the enzyme solution in a Petri dish. The Petri dish was covered with aluminum foil and vacuum infiltrated for 30 minutes and incubated another 2.5 hours at room temperature.
After digestion, protoplasts were released by gentle shaking. An equal amount of W5 solution (154mM NaCl, 125mM $CaCl_2$, 5mM KCl, 2mM MES and 5mM glucose) was added and the protoplasts were filtered with a 40µm strainer (BD Bioscience) into a round-bottomed tube on ice. Petri dished were rinsed once more with W5 solution. Finally protoplast mixtures were centrifuged in a swinging bucket centrifuge at 1000 rpm for 3 minutes. Afterwards, the supernatant was removed and the pellet was resuspended in W5 solution. Protoplasts were counted and the concentration was adjusted to 2×10^5 protoplasts per ml. The protoplasts were kept on ice for recovery during 30 minutes and were subsequently centrifuged and resuspended in the same volume of MMg solution (0.4M mannitol, 15mM $MgCl_2$ and 4mM MES).
For transformation, 10µl of 10-20µg DNA (Fig 7-18) was transferred to a round bottomed Eppendorf tube. 100µl of protoplast suspension was added and was mixed with the DNA through gently tapping. Finally 110µl 20% PEG solution (20% (w/v) PEG 4000 (Fluka), 0.2M mannitol and 0.1M $CaCl_2$) was added. After 5 minutes incubation at room temperature, the mixture was diluted with 2 volumes of W5 solution and centrifuged for 1 minute. The supernatant was removed and the protoplast pellet was transferred to a 6 well plate with 1ml of WI solution (0.5M mannitol, 4mM MES and 20mM KCl). Protoplast transformation was evaluated after 10 hours of incubation.

For transformation, a pK7WG2 vector backbone was used (Fig 7-18) with one copy of the cytoplasmic eGFP gene.

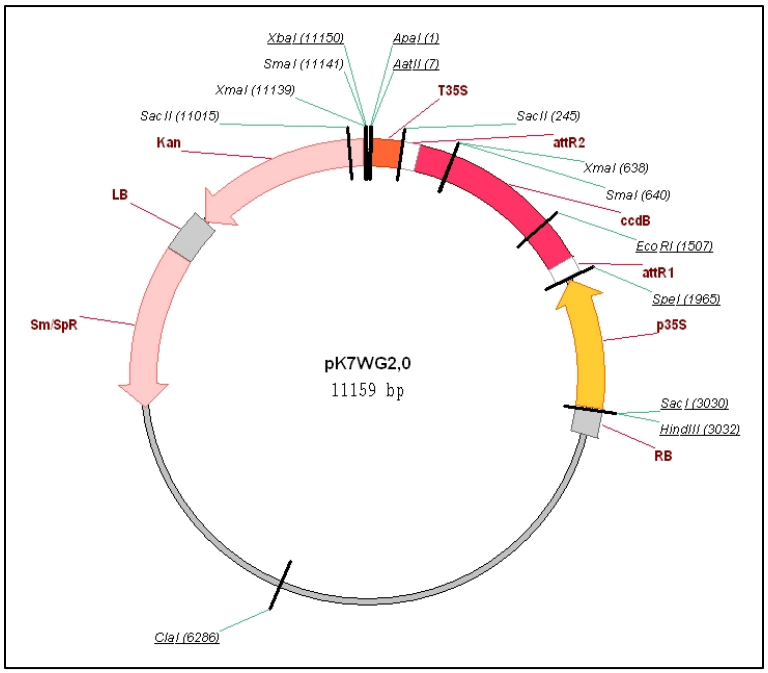

Fig 7-18 Vector map of the pK7WG2 vector backbone that was used for transformation of *Maesa lanceolata* and *Arabidopsis thaliana* protoplasts.

The pK7WG2-eGFP DNA was purified from *E. coli* using the PureYield™ Plasmid Maxiprep System from Promega. The manual can be read on-line (http://www.promega.com/tbs/tm280/tm280.pdf).

7.5.6 Microscopy

For microscopic analysis of protoplasts and evaluation of fluorescence expression in transgenic tissue, an inverted Olympus (IX-81) microscope with CellM™ software (Olympus) was used.

7.5.7 Statistics

For statistical analysis SPSS software (version 15.0) was used. For comparison of mean values between two treatments we used an independent-samples T-test and for comparison of mean values between more than two treatments a one way analysis of variance (ANOVA) was performed using a Tukey test.

CHAPTER 8 CONCLUSIONS AND FUTURE PERSPECTIVES

Maesa species are a group of saponin-producing plants, some of which have interesting medicinal properties and promising pharmaceutical applications. A myriad of biological activities have been assigned to *Maesa* saponins: e.g. insecticidal, virucidal, fungicidal and molluscicidal activities (Apers et al. 2001; Sindambiwe et al. 1998). For this research, we have collected plant material with confirmed species identity: *Maesa lanceolata, M. balansae, M. argentea* and *M. perlarius*. A subset of the *Maesa lanceolata* saponins were found to have anti-angiogenic action and could therefore be used in modern approaches of cancer treatment (Apers et al. 2002). *Maesa balansae* saponins have already been investigated thoroughly because they have a strong anti-leishmanial activity and could be used as an alternative and cheaper method for the treatment of leishmaniasis (Maes et al. 2004a; Maes et al. 2004b). *Maesa argentea* and *Maesa perlarius* are not yet investigated in detail. However, they both produce a saponin mixture that has also anti-leishmanial actions (Foubert et al. 2008; Vermeersch et al. 2009). Besides these useful biological activities associated with *Maesa* saponins, toxic or haemolytic variants have been identified. In fact, the number of saponins identified in a single species turns out to exceed the handful of molecules previously identified. The current estimation of saponins produced by *Medicago truncatula* runs well over the hundred. The purification of specific minor saponins can therefore be cumbersome. In *Maesa lanceolata*, the most promising saponins with the highest medicinal activity and the lowest cytotoxicity are often produced in very low amounts. With this PhD work, a set of studies and approaches were undertaken to investigate the biosynthesis of saponins in four *Maesa* species. Part of the PhD was conducted in the framework of COMBIPLAN, a SBO ('strategisch basis onderzoek') project. It largely comprised one of three tasks involving the development of tissue culture techniques for *Maesa* species. The project was developed on the background of previous studies that have focused on three major subjects:

- **Taxonomic and morphological studies.** *Maesa* species have originally been placed in the family of Myrsinaceae. Because of differences with other members in the family, especially in floral characteristics, it was suggested that the genus *Maesa* should be removed from the Myrsinaceae and placed in a newly defined family, Maesaceae (Anderberg and Stahl 1995; Caris et al. 2000). These studies have mainly been focused on three species: *M. argentea, M. perlarius* and *M. japonica.*
- **Development of techniques for identification and isolation of secondary metabolites.** Saponin mixtures have been identified and characterized in leaves of *M. lanceolata* and *M. balansae* (Apers et al. 1999; Leonard et al. 2003; Sindambiwe et al. 1996; Theunis et al. 2007). For *M. argentea* and *M. perlarius* saponins in twigs and leaves were observed and compared with

saponins in *M. lanceolata* and *M. balansae* (Foubert et al. 2008). Triterpene saponins were also identified in *M. laxiflora* (Jiang et al. 1999), *M. japonica* (Koike et al. 1999), *M. ramentacea* (Tuntiwachwuttikul et al. 1997) and *M. tenera* (Koike et al. 2001). In addition, benzoquinones were found and identified in fruits of *M. lanceolata* (Manguro et al. 2003; Mossa et al. 1999).

- **The study of biological activities and structure-activity relationships of saponins and benzoquinones**. The main biological activities of interest are antiangiogenic and antileishmanial action (Apers et al. 2002; Maes et al. 2004a; Maes et al. 2004b). Benzoquinones from *M. lanceolata* prove to have antibacterial, cytotoxic and antioxidant activities (Muhammad et al. 2003; Taniguchi et al. 1978).

The overall aim of COMBIPLAN was to modify the saponins biosynthesis in a combinatorial fashion. For this purpose tools were developed that could eventually also be adapted for other secondary metabolite producing plant species.

First, we fine-tuned a protocol for saponin extraction from *Maesa* species that allowed rapid and efficient extraction in small volumes. A method for saponin extraction from *Maesa* was already described in Theunis et al. (2007). However, we experienced two major disadvantages using this method: (1) large amounts of starting material were necessary and (2) the extraction protocol was very time consuming. The adapted protocol required 10 times less starting material and processing time was lowered drastically, as 60 samples could be handled in one time. Saponin extracts were analyzed using thin layer chromatography (TLC). TLC data were used for rapid and comparative analyses. For quantification and detailed identification of the different types of saponins HPLC-MS was used. The adaptation of the extraction and TLC protocol was crucial for this and future work on *Maesa* species.

The saponin content was determined for greenhouse grown and *in vitro* plants grown under different environmental conditions and after treatment with different substances that influence the development or that mimic a pathogenic attack. Saponin content in greenhouse grown and *in vitro* plants was quantitatively and qualitatively comparable, which makes *in vitro* cultures a good system to study saponin production and accumulation. TLC and HPLC data also revealed that saponin content was relatively high in roots, shoots and stems of all species and that the production did not seem to change after challenging the plants with different types of phytohormones and elicitors. Instead, the saponin content in greenhouse plants varied largely depending on the age of the plants and leaves. Thus, saponin production in *Maesa* plants is regulated developmentally and is largely unaffected by pathogen attack

and environmental or hormonal changes. *Maesa* saponins are therefore assigned to the class of constitutive secondary metabolites (phytoanticipins), distinct from conditionally synthesized secondary metabolites (phytoalexins). These results are, however, contradictory to the methyl jasmonate induction approach that was proposed in Chapter 5 for *Maesa lanceolata*. Identification of biosynthesis genes was intended through a functional genomics approach that was based on the assumption that elicitor treatment not only enhances the production of desired secondary metabolites but also activates the genes involved in the biosynthesis of such compounds. Such an approach did prove to be successful for identification of secondary metabolite biosynthesis genes in tobacco Bright yellow 2 (BY-2) cells (Goossens et al. 2003) and is likewise also effective for other plants that produce secondary metabolites in an inducible fashion, such as *Medicago truncatula* (Suzuki et al. 2005) or *Panax ginseng* (Ali et al. 2006; Zhong and Zhang 2005). A cDNA-AFLP pilot of elicited *Maesa lanceolata* shoots showed that the shoots were methyl jasmonate responsive; however, since we could not prove a coordinate upregulation of saponins, we cannot make decisive conclusions about the identity of the genes that were later on designated as 'putative saponin biosynthesis genes'. Although it is sometimes reported that upregulation of biosynthesis genes does not necessarily correlate with an increased saponin production, e.g. for *Euphorbia tirucalli* (Uchida et al. 2009). With the applied chromatography and identification methods no changes in saponin biosynthesis were detected using RNAi or overexpression constructs of *M. lanceolata* genes. This contrasted with the overexpression of *Panax ginseng* and *Bupleurum falcatum* genes which did lead to production of novel saponins in *Medicago truncatula* and *Maesa lanceolata*. Concerning RNAi silencing of endogenous *M. lanceolata* genes, it should be noted that we could not discriminate whether the negative results were obtained because the wrong genes were silenced or because the silencing itself was not successful.

For *Maesa* spp., as for many other plants producing interesting saponins, no genome and little EST sequence information is available, which makes gene identification difficult and consequently techniques need to be developed that do not require pre-existing sequence knowledge. Such a strategy is being exploited in *Avena* species that produce triterpene saponins, avenacins. Avenacins have been found to confer resistance to the root-infecting fungus *Gaeumannomyces graminis* that causes 'take-all' disease (Papadopoulou et al. 1999). A major advantage in these studies is the fluorescence of the main saponins, avenacin A-1 and B-1, because of an *N*-methylanthranilic acid residue. This property can be used to screen very fast for saponin deficient mutants by observing *N*-methylanthranilic acid fluorescence in roots (Papadopoulou et al. 1999). In this way different saponin-deficient (*sad*) mutants were found and further characterized using radioactive precursors (Trojanowska et al. 2001), segregation analysis

(Mylona et al. 2008), sequence comparisons and phylogenetic analysis (Mugford et al. 2009). Such a strategy, based on mutant analysis, would also be preferred for *Maesa* species, though; investigations would be hampered because of the lack of a suitable high-throughput screen since *Maesa* saponins are not fluorescent. The most recent publication on *Avena* saponins provides a TLC-based method for facile identification of β-amyrin synthase and cytochrome P450 enzyme mutants. In addition, using these TLC-based systems, a novel β-amyrin derivative could be detected and the impact of mutations on primary sterol biosynthesis was monitored (Qin et al. 2010). This high-throughput screen approach relies on the accumulation of generic triterpene precursors and does not depend on detection of the fluorescent end-products. In principle, this technique could therefore be applied to identify early triterpene synthesis mutants in other plants species, like *Maesa*, without any *a priori* knowledge of the pathways.

Recently, we have attempted another method for the identification of a *Maesa lanceolata* biosynthesis gene. Therefore a sequence similarity based approach was taken to isolate a putative β-amyrin synthase. Degenerate primers were constructed based on conserved regions in β-amyrin synthases identified in other plant species. Using these primers a fragment was amplified, that was further sequenced and identified as a putative *M. lanceolata* β-amyrin synthase, based on sequence comparison with characterized enzymes. Currently, the *M. lanceolata* β-amyrin synthase is being silenced using RNAi technology, to investigate the importance of this enzyme on saponin biosynthesis in *M. lanceolata*. In addition, overexpression constructs are being constructed.

A second combinatorial biochemistry approach attempted in this thesis was based on a successful example in potatoes, where novel glycoalkaloids were produced after somatic hybridization of two different *Solanum* species (Laurila et al. 1996). Before we could make somatic hybrids between different *Maesa* species, we first had to develop an efficient protocol for protoplast isolation. An isolation method was optimized for *in vitro* leaves, callus and hairy roots of all species. Fluorescent protoplasts could also be obtained when using transgenic hairy roots and callus material as explant material for isolation. When performing the different isolation tests, we found that the manual counting of protoplasts was a very labour intensive process. Therefore, we established a method for automatic counting of protoplasts using a Coulter Counter Multisizer ^{TM}. In contrast to the establishment of a protocol for protoplast isolation, we encountered great difficulties in developing a protocol for efficient protoplast regeneration. Despite the numerous experiments performed, we were not able to find the conditions that induce mitotic divisions. Cell wall formation occurred only in a small number of *Maesa argentea* leaf protoplasts and this lack of cell walls is probably the reason for the observed absence of mitosis and the

regeneration recalcitrance of the protoplasts. Further research should focus on finding conditions to allow formation of cell walls and break the recalcitrance of the protoplasts so fusions between the different species can be performed. Transient transformation of protoplasts was successful; however, the protocol still needs some optimization because transformation efficiency was rather low. A transient expression system in *Maesa* protoplasts could be used to quickly test the functionality of new constructs but also to investigate the activity of the putative saponin biosynthesis genes.

Another part of this dissertation focused on the cultivation and conservation of the four *Maesa* species *in vitro. Maesa* species are mainly growing in tropic regions of Africa and Asia; therefore, it is difficult to cultivate the plants in a moderate climate like in Belgium. Accordingly, we cannot use the *Maesa* plants for breeding and cultivation programs. Therefore, the *in vitro* plants were used for clonal propagation of *Maesa* species. Three different micropropagation protocols were developed; (1) axillary shoot induction, (2) adventitious shoot induction and (3) callus regeneration. In future experiments, the protocol for adventitious shoot induction will not only be used for rapid propagation of *Maesa* plants but also for establishment of transgenic *Maesa* plants. *Agrobacterium tumefaciens* transformation of leaf discs is based on shoot induction of infected leaves. Therefore, an efficient shoot regeneration protocol needed to be established before transformation could be contemplated. Currently, the adventitious shoot induction method is being used for regeneration of *M. lanceolata* leaf discs injected with *A. tumefaciens* bearing a silencing construct of the *M. lanceolata* β-amyrin synthase.

In vitro culture and regeneration of plants is, however, sometimes accompanied by genetic instability through a process known as somaclonal variation (Kaeppler et al. 2000; Larkin and Scowcroft 1981; Oh et al. 2007). The variability is often noticed at the ploidy level, chromosome structure, mitotic abnormalities and other cytological disorders (Radić et al. 2005). Flow cytometry was used for ploidy analysis of regenerated *Maesa* shoots. For plantlets regenerated through axillary shoot induction and adventitious shoot induction, the ploidy was identical to the control *in vitro* plants and greenhouse grown plants. In contrast, plants regenerated from callus did show polyploidization. Polyploid plants have been reported to be superior to diploids with respect to morphology, genetic adaptability and tolerance to environmental stresses (Dhooghe et al. 2010; Xiong et al. 2006). The polyploid *Maesa* plants will be closely followed up and will be compared to the control plants concerning growth characteristics and leaf morphology. In addition, we will determine cell size and perform DAPI nuclear staining.

Regenerated plantlets and *in vitro* cultures were also investigated on a biochemical level using TLC. The *Maesa* plantlets regenerated from axillary buds and adventitious shoots produced saponins in a fashion

that was reminiscent to control greenhouse and *in vitro* plants. So we can conclude that the established protocols for axillary and adventitious shoot induction produced "true to type" *Maesa* plants in terms of ploidy and saponin production. In contrast to calli, that did not produce detectable amounts of saponins, *Maesa argentea* and *M. perlarius* plants regenerated from these calli did have the capacity to produce saponins to an extent that was similar to that of control plants. So it seems that for *Maesa* differentiation is a prerequisite for triterpene saponin production. Interestingly, tetraploids have been reported to produce more secondary metabolites than the corresponding diploids (Berkov and Philipov 2002; Gao et al. 2002; Gao et al. 1996). Young *Maesa* polyploid plants did not show a higher saponin content compared to control *in vitro* and greenhouse plants. However, since not only quantitative changes but also qualitative changes have been reported for secondary metabolite production in tetraploid plants, further HPLC-MS analyses of fully grown tetraploid *Maesa* plants are needed (Berkov and Philipov 2002). The importance of polyploid plants in agriculture is well documented (Lewis 1980) and the interest is increasing in the field of medicinal plants. In this view, the spontaneous polyploidization for *Maesa* could be an interesting tool to produce superior plants in terms of growth and saponin production, instead of an undesired side-effect of *in vitro* propagation.

We have also established a protocol for *Agrobacterium rhizogenes* mediated transformation of *Maesa lanceolata*. Resulting hairy roots grew relatively fast on medium without the application of exogenous phytohormones. In addition to the classical culture conditions on solid and in liquid medium, we also upscaled the growth of hairy roots in a temporary immersion bioreactor system (TIB). As confirmed with TLC, *M. lanceolata* hairy roots produced saponins in the same quantities as roots from *in vitro* and greenhouse grown plants, however, less than shoots of the same plants. Efforts to increase the production in hairy roots to levels in leaves and shoots did not prove successful. A whole array of potential elicitors was added to hairy root cultures but none of the substances was capable of effectively inducing the saponin synthesis. First of all, this is another proof that *Maesa lanceolata* saponins are not inducible, however, these experiments give also evidence that saponin production in hairy roots is relatively stable, which makes hairy roots a robust system for further silencing and overexpression studies. The down side of the use of *in vitro* hairy root cultures is that the maintenance is labour intensive and involves culture handling with a high risk of microbial contamination and subsequent loss of original cultures (Grout 1995). The conservation of original cultures using cryopreservation technology can avoid these problems: storing samples in liquid nitrogen eliminates the need for periodic subculturing and reduces the risk of accumulation of somaclonal variation (Teoh et al. 1996). Therefore a

cryopreservation protocol, using an encapsulation-dehydration technique, was successfully established for *Maesa lanceolata* hairy roots. We also adapted the same protocol for cryopreservation of *Medicago truncatula* hairy roots. More research, however, will be needed to further characterize the root cultures that have been frozen.

Taken together, several tools have been developed that resulted in the establishment of a good platform for study of saponins in four medicinal *Maesa* species. We report here for the first time methods for *A. rhizogenes* mediated transformation and protoplastation of *Maesa* spp. However, so far, intentions to change the saponin mixture in *M. lanceolata* proved to be more difficult than expected. Though, despite the encountered setbacks, one of the experimental approaches did lead to the production of novel secondary metabolites. *In vitro* cultures and cryopreservation techniques were developed to conserve these rare and tropical plant species. *In vitro* micropropagation protocols were established for efficient propagation, hence circumventing problems with conventional propagation of *Maesa* spp. Finally, studies on saponin production learned us more about the not-inducible nature of these compounds in *Maesa.*

Summary

Maesa argentea, M. balansae, M. lanceolata and *M. perlarius* are tropical plant species that produce a mixture of pharmaceutically interesting saponins. These saponins have a myriad of biological activities, e.g. virucidal, molluscicidal, insecticidal, antileishmanial and antiangiogenic action. Though, *Maesa* saponins cannot readily be used as drugs because of their cytotoxicity. Interestingly, small structural changes can have a drastic effect on saponin action and toxicity. Hence, triterpene saponins of *Maesa* and other plant species producing similar metabolites could be used as building blocks to make new saponins with better properties in terms of pharmaceutical applicability.

The overall goal of this thesis was to develop tools to build a platform for further studies in these unfamiliar plant species. We used these tools to investigate saponin production in *Maesa* species and to modulate the saponin synthesis. In addition, we have established protocols for *in vitro* cultivation and propagation of the four selected *Maesa* species.

For rapid and comparative saponin analysis in *Maesa* species, a small-scale protocol for saponin extraction and detection using thin layer chromatography (TLC) was developed. For quantification and detailed identification of different types of saponins HPLC-MS was used (in collaboration with the Lab of Pharmacognosy and Pharmaceutical analysis – University of Antwerp). Saponin analysis of greenhouse grown and *in vitro* plants revealed that the production of saponins in *Maesa* is developmentally controlled. Treatment with substances that mimic a pathogen attack did not have an effect on saponin production in *in vitro* plants, greenhouse plants or hairy roots.

In vitro cultures were established for all four species and three different plant regeneration protocols were developed; axillary shoot induction, adventitious shoot induction and callus regeneration. Recovered shoots were analyzed on a biochemical and genetic level. Axillary and adventitious shoot formation protocols produced true-to-type plantlets in terms of ploidy and saponin content. Shoots induced on callus were polyploid. The increase in ploidy did not have an effect on the saponin content. In addition to these classical *in vitro* culture techniques, an efficient protocol for hairy root induction through *Agrobacterium rhizogenes* mediated transformation was worked out. Because periodic subculturing is very time-consuming and increases the risk on contamination, a protocol for long term storage of hairy roots at -196°C was developed using an encapsulation-dehydration technique.

Within this work also two combinatorial biosynthesis approaches are proposed for modulation of the saponin production in *Maesa* species without any pre-existing genomic knowledge. The first method was based on suppression of putative endogenous saponin biosynthesis genes and overexpression of putative saponin biosynthesis genes from other plant species producing similar saponins. The silencing approach did not prove to be successful; however, novel saponins were produced following overexpression of heterologous genes. For the second approach we intended to perform somatic hybridization of different *Maesa* species through protoplast fusion. An efficient method for protoplast isolation and counting was established. Though, *Maesa* protoplasts were recalcitrant with respect to regeneration because their inability to form a novel cell wall. Transient transformation of these protoplasts was possible; however, further optimization of the technique will be necessary to turn this into a useful transformation technology.

Samenvatting

Maesa argentea, M. balansae, M. lanceolata en *M. perlarius* zijn tropische planten die een waaier aan farmaceutisch belangrijke saponines produceren. Deze saponines hebben verschillende biologische activiteiten, zoals virucidale, molluscicidale, antileishmaniale en antiangiogene werking. *Maesa* saponines kunnen echter niet zomaar gebruikt worden als geneesmiddelen omdat ze ook cytotoxisch zijn. Uit voorafgaand onderzoek bleek reeds dat kleine structurele veranderingen een drastisch effect kunnen hebben op de activiteit en toxiciteit van saponines. Bijgevolg zouden triterpenoïde saponines van *Maesa* species of andere planten die gelijkaardige saponines produceren, kunnen dienen als 'bouwblokken' om nieuwe saponines te maken met betere eigenschappen voor farmaceutische toepassingen.

Het hoofddoel van deze thesis was het ontwikkelen van technologieën of 'tools' om een soort 'platform' te maken voor verdere studies in deze plant species. De ontwikkelde tools hebben we dan gebruikt om saponineproductie in *Maesa* species te onderzoeken en om saponinebiosynthese te moduleren. Daarnaast hebben we ook protocols ontwikkeld voor *in vitro* cultivatie en vermeerdering van de vier geselecteerde *Maesa* species.

Een protocol voor saponine-extractie en -detectie, door middel van dunne laag chromatografie (TLC), werd op punt gesteld. Op die manier konden we snel een idee hebben van de saponine-inhoud in verschillende species, organen en weefsels. Voor kwantitatieve data en gedetailleerde identificatie van verschillende types van saponines werd HPLC-MS gebruikt (in samenwerking met het labo voor Farmacognosie en farmaceutische analyse – Universiteit Antwerpen). Vergelijkende analyse van saponines in serreplanten en *in vitro* planten toonde dat de productie van saponines voornamelijk gestuurd werd door de ontwikkeling van de planten. Behandeling met elicitoren had geen effect op saponineproductie in *in vitro* planten, serreplanten of hairy roots.

In vitro culturen werden opgestart voor alle vier de *Maesa* species en drie verschillende regeneratieprotocols werden ontwikkeld; okselknop scheutinductie, adventief scheutinductie en callusregeneratie. Door middel van TLC werd in the geregeneerde planten de saponine-inhoud onderzocht en door middel van flow cytometrie werd ook het ploïdieniveau van de planten bestudeerd. Okselknop en adventief scheutregeneratie protocols leidden tot 'true-to-type' planten wat betreft ploïdie en saponine-inhoud. Scheuten die geïnduceerd werden op callus, waren daarentegen polyploïd.

Het verhoogde ploidielevel had echter geen effect op saponineproductie. Bovenop deze klassieke *in vitro* technieken, hebben we een efficiënt protocol voor hairy root inductie, door middel van *Agrobacterium rhizogenes* gemedieerde transformatie, uitgewerkt. Omdat periodisch subculturen van hairy roots tijdrovend is en het risico op contaminatie verhoogt, werd ook een protocol ontwikkeld voor langetermijnbewaring van hairy roots in vloeibare stikstof (-196°C). Voor efficiënte cryopreservatie werd gebruik gemaakt van een encapsulatie-dehydratatie techniek.

In dit werk werden ook twee methodes voor modulatie van saponineproductie in *Maesa* species voorgesteld. De eerste methode was gebaseerd op onderdrukking of 'silencing' van mogelijke endogene saponinebiosynthese genen en overexpressie van mogelijke saponinebiosynthese genen uit andere planten die gelijkaardige saponines produceren. In tegenstelling tot de silencing methode, werden bij de overexpressie aanpak nieuwe saponines geproduceerd. Een tweede methode om saponineproductie te veranderen was gebaseerd op somatische hybridisatie van verschillende *Maesa* species door middel van protoplastfusie. Een efficiente methode voor isolatie en automatisch tellen van protoplasten werd op punt gesteld. *Maesa* protoplasten bleken echter recalcitrant op vlak van regeneratie. Deze recalcitrantie wordt waarschijnlijk veroorzaakt door hun onvermogen om een nieuwe celwand te maken. Transiënte transformatie van deze protoplasten was mogelijk maar enkel een klein aantal cellen kon getransformeerd worden. Bijgevolg zal verdere optimalisatie nodig zijn om gebaseerd op dit protocol een 'high-throughput' transformatie technologie voor *Maesa* species te ontwikkelen.

Addendum I Complete list of all compounds tested for elicitation of saponin production in *Maesa in vitro* cultures. Saponin content in hairy roots and the corresponding culture medium are presented in percentage of dry weight and volume percent, respectively.

TREATMENT	ELICITOR	CONCENTRATION	TISSUE	SPECIES	DURATION	Saponin concentration (%)	
						Hairy roots	Culture medium
Control	**-**	-	Hairy root	*M. lanceolata*	-	2.1	0.0040
Controlled infection	**Chitosan**	50 mg/l	Hairy root	*M. lanceolata*	1 week	-[a]	-[a]
		100 mg/l				-[a]	-[a]
		150 mg/l				-[a]	-[a]
		200 mg/l				-[a]	-[a]
		250 mg/l				-[a]	-[a]
	Fungal spores	5000 spores/ml	Hairy root	*M. lanceolata*	48 hours	1.3	0.0005
		10 000 spores/ml				0.9	0.0005
		50 000 spores/ml				2.6	0.0007
		100 000 spores/ml				2.2	0.0027
	Mycelium homogenates	OD 0.2	Hairy root	*M. lanceolata*	48 hours	2.5	0.0010
		OD 0.3				2.9	0.0011
		OD 0.4				1.3	0.0022
		OD 0.5				2.7	0.0020
		OD 1.0				1.7	0.0014
	Yeast extract	0.5 mg/ml	Hairy root	*M. lanceolata*	48 hours	1.9	0.0030
		4.0 mg/ml				1.2	0.0011
		5.0 mg/ml				1.8	0.0015
		7.5 mg/ml				2.7	0.0028
		10.0 mg/ml				1.8	0.0015
Acid treatment	**Acetic acid**	15 mM	Hairy root	*M. lanceolata*	7 days	-[a]	-[a]
		10 mM	In vitro shoot	*M. argentea*	48 hours	-[b]	-[c]
				M. balansae		-[b]	-[c]
				M. lanceolata		-[b]	-[c]
				M. perlarius		-[b]	-[c]
		10 mM	In vitro shoot	*M. lanceolata*	0 hours	-[b]	-[c]

					8 hours	-[b]	-[c]
					24 hours	-[b]	-[c]
					48 hours	-[b]	-[c]
					56 hours	-[b]	-[c]
					72 hours	-[b]	-[c]
	Citric acid	15 mM	Hairy root	*M. lanceolata*	7 days	-[a]	-[a]
		10 mM	In vitro shoot	*M. argentea*	48 hours	-[b]	-[c]
				M. balansae		-[b]	-[c]
				M. lanceolata		-[b]	-[c]
				M. perlarius		-[b]	-[c]
	Formic acid	10 mM	In vitro shoot	*M. argentea*	48 hours	-[b]	-[c]
				M. balansae		-[b]	-[c]
				M. lanceolata		-[b]	-[c]
				M. perlarius		-[b]	-[c]
Hormone treatment	**Methyl jasmonate (MeJA)**	0.1 mM	Hairy root	*M. lanceolata*	48 hours	2.5	0.0007
		0.5 mM				1.4	0.0014
		1.0 mM				2.8	0.0082
		10 mM				2.7	0.0055
		15 mM				2.3	0.0065
		0.1 mM	In vitro shoot	*M. argentea*	48 hours	-[b]	-[c]
				M. balansae		-[b]	-[c]
				M. lanceolata		-[b]	-[c]
				M. perlarius		-[b]	-[c]
	2,4-dichlorofenoxyacetic acid (2,4-D)	0.1 mM	Hairy root	*M. lanceolata*	48 hours	1.7	0.0007
		0.01 mM	In vitro shoot	*M. argentea*	48 hours	-[b]	-[c]
				M. balansae		-[b]	-[c]
				M. lanceolata		-[b]	-[c]
				M. perlarius		-[b]	-[c]
	Naphthylphtalamic acid (NPA)	0.01 mM	Hairy root	*M. lanceolata*	72 hours	2.7	0.0008
	Salicylic acid (SA)	0.1 mM	Hairy root	*M. lanceolata*	48 hours	0.8	0.0050
		0.2 mM				0.7	0.0052
		0.01 mM	In vitro shoot	*M. argentea*	48 hours	-[b]	-[c]

			M. balansae		-[b]	-[c]
			M. lanceolata		-[b]	-[c]
			M. perlarius		-[b]	-[c]
	Benzothiadiazole (BTH)	0.1 mM	Hairy root	*M. lanceolata*	48 hours	1.8	0.0006
		1.0 mM				1.8	0.0006
		10 mM				1.2	0.0005
		50 mM				1.4	0.0004
	Benzyladenine (BA)	0.9 µM	Hairy root	*M. lanceolata*	48 hours	2.2	0.0012
	Abscisic acid (ABA)	0.1 mM	Hairy root	*M. lanceolata*	48 hours	1.2	0.0003
		0.01 mM	In vitro shoot	*M. argentea*	48 hours	-[b]	-[c]
				M. balansae		-[b]	-[c]
				M. lanceolata		-[b]	-[c]
				M. perlarius		-[b]	-[c]
	Gibberellic acid (GA3)	0.01 mM	In vitro shoot	*M. argentea*	48 hours	-[b]	-[c]
				M. balansae		-[b]	-[c]
				M. lanceolata		-[b]	-[c]
				M. perlarius		-[b]	-[c]
	Methyl jasmonate (MeJA) + 2,4-dichlorofenoxyacetic acid (2,4-D)	0.1 mM + 0.1 mM	Hairy root	*M. lanceolata*	48 hours	2.8	0.0008
	Methyl jasmonate (MeJA) + Naphthylphtalamic acid (NPA)	0.1 mM + 0.01 mM	Hairy root	*M. lanceolata*	48 hours	1.5	0.0019
	Methyl jasmonate (MeJA) + Abscisic acid (ABA)	0.1 mM + 0.1 mM	Hairy root	*M. lanceolata*	48 hours	1.03	0.0032
	Naphthylphtalamic acid (NPA) + Salicylic acid (SA)	0.01 mM + 0.1 mM	Hairy root	*M. lanceolata*	48 hours	2.2	0.0015
		0.01 mM + 0.2 mM				1.8	0.0032
Permeabilization/ Cell lysis	**$CdCl_2$**	0.1 mM	Hairy root	*M. lanceolata*	48 hours	2.1	0.0010
		1.0 mM				3.9	0.0056
		2.0 mM				2.0	0.0055
		5.0 mM				2.1	0.0027

$CaCl_2$	0.1 mM	Hairy root	*M. lanceolata*	48 hours	2.5	0.0023
	5.0 mM				2.9	0.0009
	10 mM				2.2	0.0004
	15 mM				1.6	0.0003
	20 mM				2.3	0.0012
	100 mM				2.2	0.0007
H_2O_2	0.1 mM	Hairy root	*M. lanceolata*	48 hours	1.6	0.0003
	0.5 mM				1.8	0.0019
	1.0 mM				2.5	0.0017
	5.0 mM				3.3	0.0031
EDTA	0.1 mM	Hairy root	*M. lanceolata*	48 hours	1.7	0.0008
Tween 20	3%	Hairy root	*M. lanceolata*	48 hours	1.7	0.0016
Triton X-100	3%	Hairy root	*M. lanceolata*	48 hours	1.6	0.0002
Dimethylsulfoxide (DMSO)	0.1%	Hairy root	*M. lanceolata*	48 hours	2.4	0.0040
	0.5%				0.9	0.0002
	1.0%				2.5	0.0018

[a] Saponin content determined using a semi-quantitative HPLC-MS technique

[b] Saponin content analysed with TLC

[c] Saponin content not analysed

Addendum II Complete list of all conditions tested for regeneration of *Maesa* protoplasts. All protoplast isolations were performed overnight and incubated in the dark at 25°C, unless indicated otherwise.

Explant	Culture type	Plant species	Protoplast density	Salts	Sugars	Sugar concentrations	Hormones	Hormone concentrations	Other remarks
Leaf material	**Liquid**	MA, MB	4-5.10^4	MS	Sucrose Mannitol	90 mM 0.5 M *Gradual reduction*	GA3 2.4-D	0.3, 0.6, 0.9 µM 2.5, 5.0, 10 µM	Mannitol concentration reduced every week or every three days
		MA	5.10^5	MS	Glucose Sucrose	0.4 M 117 mM	2.4-D NAA Zea	0.9, 5 µM 0.54, 5.4 µM 2.3 µM	-
		MA	4-5.10^5	MS	Glucose Sucrose	0.4 M 117 mM	2.4-D Zea	1.25, 2.5, 3.75, 5, 6.25, 7.5, 8.75, 10, 11.25, 12.5 µM 1.2, 2.3, 3.5, 4.6, 5.8, 6.9 µM	-
		MA	4-5.10^5	MS	Glucose Sucrose	0.2, 0.4, 0.6, 0.8, 1 M 0.2, 0.4, 0.6, 0.8, 1 M	2.4-D Zea	7.5 µM 4.6 µM	-
		MA, MB, ML, MP	6-7.10^5	MS SH B5 KM	Glucose Sucrose	0.4 M 117 mM	2.4-D Zea	5 µM 2.3 µM	-
		MA, MB, ML, MP	6.10^5	MS	Glucose Sucrose	0.4 M 117 mM	2.4-D NAA IBA IAA Kin Zea BA NPA	5 µM 2.7 µM 5 µM 3 µM 0.5 µM 4.6 µM 4.5 µM 5 µM	-
		MA, ML	5.10^5	MS	Glucose Sucrose	0.4 M 117 mM	2.4-D NAA Kin Zea BA NPA	5 µM 2.7 µM 0.5 µM 4.6 µM 4.5 µM 5 µM	Protoplast isolation during 5 hours
		ML	2.10^4	Heller	Sucrose Mannitol	10 mM 0.4 M	NAA BA	10 µM 4.5 µM	Different types of enzymes for isolation
		ML	2.10^4	Heller	Sucrose Mannitol	10 mM 0.4 M	NAA BA	10 µM 4.5 µM	Addition of 0.1 and 0.5% Pluronic
		ML	3-4.10^5	WPM	Sucrose	30 mM	NAA	5 µM	Incubation at 25°C in 18/6h

				Mannitol	0.7 M	BA	1 µM	light/dark conditions
	MA, ML	5.10^4	MS	Glucose Sucrose	0.4 M 117 mM	2.4-D Kin	5 µM 0.5 µM	Incubation at 25°C and 28°C in 18/6h light/dark conditions
	ML	4.10^4	MS	Glucose Sucrose	0.4 M 117 mM	2.4-D Kin	5 µM 0.5 µM	Incubation at 28°C in 18/6h light/dark conditions Addition of 0.1% Pluronic
	ML	4.10^4	MS	Glucose Sucrose	0.4 M 117 mM	2.4-D Zea	5 µM 0.46 µM	Plates coated with 5% or 100% fetal calf serum
	MA, ML	4.10^4	MS	Glucose Sucrose	0.4 M 117 mM	2.4-D NAA IAA Zea TDZ Kin	5 µM 5 µM 5 µM 0.5 µM 0.5 µM 0.5 µM	Plates coated with 5% fetal calf serum
	MA, ML	5.10^4	MS	Sucrose Glucose Mannitol Sorbitol Xylose Fructose	0.08, 0.4 M 0.3, 0.5, 0.7 M 0.4, 0.5, 0.7 M 0.5, 0.7 M 0.4, 0.5, 0.6, 0.9 M 0.3, 0.5, 0.7 M	2.4-D Zea	7.5 µM 4.6 µM	Plates coated with 5% fetal calf serum
	ML	4.10^4	MS	Glucose Sucrose	0.4 M 117 mM	2.4-D Zea	5 µM 0.5 µM	Protoplast isolation during 5 hours
	ML	7.10^4	MS B5 KM SH Heller WPM	Mannitol Sucrose	0.5, 0.7 M 90 mM	2.4-D Zea	5 µM 0.5 µM	Plates coated with 5% fetal calf serum
	ML	9.10^4	B5	Mannitol Sucrose	0.5 M 90 mM	GA3 2.4-D NAA Zea	1.5, 3, 4.5, 6 µM 5 µM 5 µM 4.6 µM	Plates coated with 5% fetal calf serum
	ML	9.10^4	B5	Mannitol Sucrose	0.5 M 90 mM	GA4-7 2.4-D NAA Zea	1.5, 3, 4.5, 6 µM 5 µM 5 µM 4.6 µM	Plates coated with 5% fetal calf serum
	MA, ML	5.10^4	B5	Mannitol Sucrose	0.5 M 90 mM	IAA TDZ	3, 6, 9, 12 µM 0.45, 0.9, 4.5 µM	-
Solid	MA	$4\text{-}6.10^5$	MS	Glucose Sucrose	0.4 M 117 mM	*(protoplasts)* 2.4-D NAA	 1 µM 5.4 µM	1.2% low melting agarose On Millipore filter (0.22µm)

						Zea *(solid)* 2.4-D Zea	2.3 µM 9 µM 0.5 µM
	MB, MP	5-6.10^5	MS	Glucose Sucrose	0.4 M 117 mM	*(protoplasts)* 2.4-D NAA Zea *(solid)* 2.4-D Zea	 1 µM 5.4 µM 2.3 µM 9 µM 0.5 µM	1.2% low melting agarose On Millipore filter (0.8µm)
	MA	4-5.10^5	MS	Glucose Sucrose	0.4 M 117 mM	*(protoplasts)* 2.4-D NAA Zea *(solid)* 2.4-D Zea	 5 µM 0.54 µM 2.3 µM 0.5, 5, 9 µM 0.5 µM	1.2% low melting agarose On Millipore filter (0.8µm)
	MB, ML	8.10^5	MS	Glucose Sucrose	0.4 M 117 mM	(*protoplasts)* 2.4-D NAA Zea *(solid)* 2.4-D Zea	 5 µM 0.54 µM 2.3 µM 5 µM 0.5 µM	1.2% low melting agarose Nurse cells = MB, MP and ML callus cells (10%) On Millipore filter (0.8µm)
	MB, ML	4.10^4	B5	Mannitol Sucrose	0.5 M 90 mM	2.4-D Zea	5, 10, 15 µM 0.46, 4.6 µM	1.2% low melting agarose
	ML	2.10^4	Heller	Sucrose Mannitol	10 mM 0.4 M	NAA BA	10 µM 4.5 µM	1.2% low melting agarose
	ML	2.10^4	Heller	Sucrose Mannitol	10 mM 0.4 M	NAA BA	10 µM 4.5 µM	1.2 % low melting agarose Addition of 0.1% Pluronic
Agarose beads	MA	3-8.10^5	MS	Glucose Sucrose	0.4 M 117 mM	2.4-D Zea	2.5, 5, 7.5 µM 2.5 µM	1.2% low melting agarose
	MA, ML	4-5.10^5	MS	Glucose Sucrose	0.4 M 117 mM	2.4-D Zea	7.5 µM 4.6 µM	0.8, 1, 1.2% low melting agarose
	MA, MB, ML, MP	6.10^5	MS SH B5 KM	Glucose Sucrose	0.4 M 117 mM	2.4-D NAA Zea	5 µM 0.5 µM 2.3 µM	1.2% low melting agarose
	ML	2.10^4	Heller	Sucrose Mannitol	10 mM 0.4 M	NAA BA	10 µM 4.5 µM	1.2% low melting agarose
	ML	2.10^4	Heller	Sucrose	10 mM	NAA	10 µM	1.2% low melting agarose

				Mannitol	0.4 M	BA	4.5 µM	Addition of 0.1% Pluronic
Agarose layer	ML	2.10^4	Heller	Sucrose Mannitol	10 mM 0.4 M	NAA BA	10 µM 4.5 µM	1.2% low melting agarose
	ML	2.10^4	Heller	Sucrose Mannitol	10 mM 0.4 M	NAA BA	10 µM 4.5 µM	1.2% low melting agarose Addition of 0.1% Pluronic
Feeder layer	MA	$4\text{-}6.10^5$	MS	Glucose Sucrose	0.4 M 117 mM	*(protoplasts)* 2.4-D NAA Zea *(feeder)* 2.4-D Zea	 1 µM 5.4 µM 2.3 µM 9 µM 0.5 µM	1.2% low melting agarose Nurse cells = MB, MP and ML callus cells (10%) On Millipore filter (0.22µm)
	MB, MP	$5\text{-}6.10^5$	MS	Glucose Sucrose	0.4 M 117 mM	*(protoplasts)* 2.4-D NAA Zea *(feeder)* 2.4-D Zea	 1 µM 5.4 µM 2.3 µM 9 µM 0.5 µM	1.2% low melting agarose Nurse cells = MB, MP and ML callus cells (10%) On Millipore filter (0.8µm)
	MA	$4\text{-}5.10^5$	MS	Glucose Sucrose	0.4 M 117 mM	*(protoplasts)* 2.4-D NAA Zea *(feeder)* 2.4-D Zea	 5 µM 0.54 µM 2.3 µM 0.5, 5, 9 µM 0.5 µM	1.2% low melting agarose Nurse cells = MB, MP and ML callus cells (10%) On Millipore filter (0.8µm)
	MA	$4\text{-}6.\ 10^5$	MS	Glucose Sucrose	0.4 M 117 mM	*(protoplasts)* 2.4-D NAA Zea *(feeder)* 2.4-D Zea	 1 µM 5.4 µM 2.3 µM 9 µM 0.5 µM	1.2% low melting agarose Nurse cells = MB, MP and ML callus cells (10%) Feeder layer prepared 1 week before regeneration On Millipore filter (0.8µm)
	MB, ML	8.10^5	MS	Glucose Sucrose	0.4 M 117 mM	(*protoplasts)* 2.4-D NAA Zea *(feeder)* 2.4-D Zea	 5 µM 0.54 µM 2.3 µM 5 µM 0.5 µM	1.2% low melting agarose Nurse cells = MB, MP and ML callus cells (10%) On Millipore filter (0.8µm)
Calcium-alginate beads	ML	2.10^4	Heller	Sucrose Mannitol	10 mM 0.4 M	NAA BA	10 µM 4.5 µM	30 g/l Sodium Alginate 7.4 g/l $CaCl_2.2H_2O$

Seedlings	**Liquid**	ML	2.10^4	MS	Glucose Sucrose	0.4 M 117 mM	2.4-D NAA IAA Zea TDZ Kin	5 µM 5 µM 5 µM 0.5 µM 0.5 µM 0.5 µM	3 week-old seedlings
Callus	**Liquid**	MB, ML	5.10^5	MS SH B5 KM	Glucose Sucrose	0.4 M 117 mM	2.4-D NAA Zea	1 µM 5.4 µM 2.3 µM	
		ML	5.10^5	MS	Glucose Sucrose	0.5 M 30 mM	2.4-D	5 µM	Addition of 27 µM glycine, 1 mM glutamine and 500 mg/l casein hydrolysate
	Feeder layer	MB, MP	$5\text{-}6.10^5$	MS	Glucose Sucrose	0.4 M 117 mM	*(protoplasts)* 2.4-D NAA Zea *(feeder)* 2.4-D Zea	 1 µM 5.4 µM 2.3 µM 9 µM 0.5 µM	1.2% low melting agarose Nurse cells = MB, MP and ML callus cells (10%) On Millipore filter (0.8µm)
Hairy roots	**Liquid**	ML	$3.\ 10^5$	SH	Glucose Sucrose	0.4 M 117 mM	2.4-D NAA Zea	1 µM 5.4 µM 2.3 µM	
		ML	$1\text{-}2.\ 10^5$	MS	Glucose Sucrose	0.4 M 117 mM	2.4-D NAA Zea	1 µM 5.4 µM 2.3 µM	

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Ellen

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Personal data

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Nationality:	Belgian
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Education

2006 – 2010 **Doctoral training program** (Doctoral School of Bioscience Engineering)
→ Extra regular courses: *Metabolic Engineering*, *Molecular Breeding*, *Experimental Plant Biotechnology*, *Developmental Biology of Plants*, *Hormonal Regulation of Plant Growth*
→ Transferable skills: *Advanced Academic English: writing skills and conference skills*, *Creative Thinking*

2002 – 2006 Bachelor and Master in Biomedical Sciences, option Regenerative Medicine
Faculty of Medicine, University of Ghent
Master thesis: **Study of α-actin mutants that cause the muscular disorder nemaline myopathy**, Department of Biochemistry, Faculty of Medicine, University of Ghent
Supervised by Prof. Dr. Christophe Ampe

1996 – 2002 Secondary education: Latin – Sciences, O.L.V.-ten-Doorn College, Eeklo

Professional career

2006 – 2010 PhD fellowship
Lab of In Vitro Biology and Horticulture, Department of Plant Production, Faculty of Bioscience Engineering, University of Ghent
PhD thesis: **Development of tools for the *in vitro* conservation and modulation of saponin production of four medicinal *Maesa* species**
Supervised by Prof. Dr. Danny Geelen – due for submission **December 15th 2010**

Research related activities

Assisting practical courses

- Hormonal Regulation of Plant Growth
- Plant Biotechnology
- In Vitro and In Vivo Control of Growth Processes

Tutor for bachelor and master students

- Stefaan Goossens (2006 – 2007) Cryopreservatie van de medicinale plant *Maesa lanceolata*. Thesis KaHo Sint-Lieven, Gent
- Dieter Deryckere (2007 – 2008) Vorming van transgene en hybride cellijnen en analyse van saponinebiosynthese bij *Maesa* species. Thesis Ugent, Faculty of Bioscience Engineering
- Inge Verstraeten (2007 – 2008) Saponine-biosynthese in hairy root culturen van de medicinale plant *Maesa lanceolata*. Thesis Ugent, Faculty of Bioscience Engineering
- Yannick Demey (2007 – 2008) *In vitro* cultuur en cryopreservatie van *Maesa lanceolata* en *Medicago truncatula*. Thesis Katho, Roeselare
- Wei Lei (2008 – 2009) Somatic hybridization of protoplasts of *Maesa* species. Thesis Ugent, Faculty of Bioscience Engineering
- Goutam Barua (2009 – 2010) Protoplast fusion in *Cichorium intybus* L. Thesis Ugent, Faculty of Bioscience Engineering
- Nathalie Van Den Bleeken (2009-2010) Elicitatie van triterpenoïde saponinen in *in vitro* en *in vivo Maesa* culturen. Thesis Ugent, Faculty of Bioscience Engineering
- Brecht Lootens (2009 – 2010) *In vitro* propagatie en protoplastcultuur van de medicinale plant *Maesa.* Thesis KaHo Sint-Lieven, Gent

International conference contributions

- International Cryopreservation Symposium
  February 21 – 23, 2008, Oulu, Finland
  Oral presentation
- 1st International Symposium on Cryopreservation in Horticultural Species
  April 5 – 8, 2009, Heverlee, Belgium
  Oral presentation
- 4th International Symposium Breeding Research on Medicinal and Aromatic Plants (ISBMAP2009)
  June 17 – 21, 2009, Ljubljana, Slovenia
  Oral presentation

National conference contributions

- 13th PhD symposium on Applied Biological Sciences
  October 17, 2007, Leuven, Belgium
  Poster presentation
- National meeting on Epigenetics and Somaclonal Variation (BPBA)
  November 23, 2007, Gembloux, Belgium
  Poster presentations
- National meeting on Secondary Metabolites and Molecular Farming (BPBA)
  November 7, 2008, Ghent, Belgium
  Poster presentation
- National meeting on Plant Hormones (BPBA)
  November 13, 2009, Gembloux, Belgium
  Poster presentation

A1 Publications

Lambert E., Goossens A., Panis B., Van Labeke M.C. and Geelen D. (2009) Cryopreservation of hairy root cultures of *Maesa lanceolata* and *Medicago truncatula*. Plant Cell Tissue and Organ Culture, 96:289-296

Lambert E. and Geelen D. (2010) High efficiency protoplast isolation from *in vitro* cultures and hairy roots of *Maesa lanceolata*. African Journal of Biotechnology, 9(42):7071-7078

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Faizal A., Lambert E., Foubert K., Apers S. and Geelen D. (2010) *In vitro* propagation of four saponin producing *Maesa* species. Plant Cell Tissue and Organ Culture. Published online (DOI 10.1007/s11240-010-9909-2).

Vandamme D., Lambert E., Waterschoot D., Tondeleir D., Vandekerckhove J., Machesky L.M., Constantin B., Rommelaere H. and Ampe C. (2009) Phenotypes induced by NM causing α-skeletal muscle actin mutants in fibroblasts, Sol 8 myoblasts and myotubes. BMC Research Notes, 2(40)

Vandamme D., Rommelaere H., Lambert E., Waterschoot D., Vandekerckhove J., Constantin B. and Ampe C. (2009) α-skeletal muscle actin mutants causing different congenital myopathies induce similar cytoskeletal defects in cell line cultures. Cell Motility and the Cytoskeleton, 66(4):179-192

Vandamme D., Lambert E., Waterschoot D., Cognard C., Vandekerckhove J., Ampe C., Constantin B. And Rommelaere H. (2009) α-skeletal muscle actin nemaline myopathy mutants cause cell death in cultured muscle cells .Biochimica et Biophysica Acta, 1793(7):1259-1271

Language and computer skills

Dutch: Mother tongue, **English:** Fluent, **French:** Good, **German:** Basic, **Spanish:** Basic

Computer skills: Knowledge of typing
Office 2003/2007
Sigma Plot, SPSS, Vector NTI, EndNote

Personal interests

Literature and poetry, languages (Spanish), sports (running, swimming, squash, karate), music
Active for 8 years in a youth movement as monitor (of which several years as the main responsible)